THE EFFECT OF AGITATION ON BREWERS' YEAST (*SACCHAROMYCES CEREVISIAE*) QUALITY

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SYNOPSIS

In beer brewing, yeast, mainly of the genus *Saccharomyces*, is used to convert sugars in cereal-based extracts to ethanol, carbon dioxide and organoleptic compounds. The process can be split into three sub processes namely: wort preparation, fermentation and yeast handling, and post-fermentation treatments (Basson, 1996). The yeast-handling sub-process involves the removal of yeast from the fermenter (cropping) for re-use after fermentation is complete. During cropping, yeast is transferred via a heat exchanger, where it is cooled from 14°C to 4°C, to a collection vessel. The cooled yeast is stored in the collection vessel for between 4 to 24 hours after which it is re-used to inoculate the next fermentation. During yeast storage, the yeast suspension is agitated to maintain suspension homogeneity and prevent the occurrence of hotspots within the slurry. The vessel is situated in a temperature-controlled room at 4°C is operated under anaerobic conditions to hold the yeast in a metabolically inactive state. Despite these precautions, it is hypothesised that storing the yeast suspension in the YCV can lead to physiological and mechanical stresses which could have an adverse effect on its quality.

Although there are two possible stresses operating in the YCV, this study will mainly focus on mechanical stress. Mechanical stress by agitation can affect the yeast quality by release of haze forming materials through mechanical abrasion (Lewis and Poerwantaro, 1991). Agitation can also lead to loss of quality is by a loss of membrane integrity which leads to the release of extracellular proteases and a loss of viability (McCaig and Bendiak, 1985a). The level of stress that the yeast cells in a suspension is subjected to is dependent on the intensity and duration of agitation, the concentration of the suspension, the rheology of the fluid, the physiological state of the cells and the storage temperature. The objectives of this study are as follows: to

- Classify the rheology of yeast suspensions at different concentrations
- Quantify mixing in a stirred tank reactor
- Link mixing in stirred tank reactors with the rheology of yeast suspensions
- Investigate the effect of agitation on yeast quality indicators such as membrane integrity, vitality and the amount of haze generated.
- Recommend optimum agitation rate, duration of agitation and storage temperature.

The yeast suspension used in this study was the brewer's yeast, *Saccharomyces cerevisiae* SAB 5 which was obtained from the YCV of the SABMiller Brewery in Newlands (Cape Town, South Africa). The yeast collection vessel is replicated on laboratory scale and the slurry subjected to varying agitation rates ranging from 200 to 800 rpm (between 0.72 and 2.92 ms^{-1}), using the Rushton turbine and 45° pitched blade impeller types, at cell concentrations of 30 to 70% wet weight and temperatures of 4 and 14°C. Analytical techniques, which quantify yeast quality, were then used to measure the quality of the agitated yeast. The analytical techniques used to quantify yeast quality are; methylene blue staining assay for quantification

of viability, protease assay for minor membrane damage, haze analysis for minor cell wall damage and small-scale fermentation to quantify the metabolic activity of the stressed cells.

In order to have a better understanding of the degree of homogeneity observed in the laboratory scale YCV, a tracer consisting of 3M KCl was injected as a pulse at time zero and the conductivity of the resulting mixture observed over a period of time to determine mixing and circulation times. Mixing time was determined as the time taken at which the conductivity data had a standard deviation of between 5-10% of the mean.

The following results were obtained:

- The rheology of the yeast suspension was found to be dependent on the suspension concentration. In the lower concentration ranges (< 20% wet weight), the rheology of the yeast suspension was found to be Newtonian and pseudoplastic in the concentration ranges above 20% wet weight. The rheograms were fitted with different models and at yeast concentrations between 20 and 44.0% wet weight, the power law model fitted the data whereas at higher yeast concentrations (58.0 to 66.3% wet weight), the Herschel-Bulkley model gave the best fit. The relationship between the apparent viscosity and suspension concentration was found to be exponential. In conclusion, the nature of the rheology of the yeast suspension as well as magnitude of the apparent viscosity is a function of its concentration.

- A relationship between the rheology of the yeast suspension and mixing time was established by investigating the effect of suspension concentration on mixing time. The relationship between mixing time and yeast suspension concentration was found to be exponential and so was the relationship between yeast suspension concentration and apparent viscosity. Hence in the concentration range investigated, the change in mixing time can be correlated largely with apparent yeast viscosity at agitation rates greater than 0.72 ms^{-1}. An investigation into the effect of agitation rate on mixing was conducted over a range of suspension concentrations. At suspension concentrations below 32% wet weight, mixing time is independent of agitation rate however, as the cell concentration exceeded 37% wet weight, mixing time became increasingly dependent on agitation rate. Of the two impeller types, the Rushton impeller gives better mixing than the pitched blade.

- The effect of the intensity of agitation on yeast quality was investigated and it was concluded that the more intense the agitation, the more pronounced the loss of viability and loss of membrane integrity. Subsequent fermentations become less successful if the impeller speed is increased above 400 rpm and if Rushton turbine is used. The amount of haze released into suspension has a peak at 600 rpm. This effect was found to be more pronounced at higher yeast suspension concentrations (> 60% wet weight).

- It was observed that the longer the period of agitation, the more pronounced the loss of yeast quality and this is highly dependent on the concentration of the yeast suspension.

- In order to determine the effect of dilution of the yeast suspension during storage on yeast quality partially diluted and undiluted yeast suspensions were subjected to mechanical stress. It is clearly seen from the results obtained that the yeast quality deteriorates as the suspension concentration of the storage phase increases. The loss of yeast quality can be attributed to an increase in the number of cells present in an aliquot of suspension which increased the rate of agitation of inter-particle interactions as well as a reduction in the efficiency of mixing as the concentration increases. Finally, there was increasing ethanol concentration which is toxic to yeast cells as the suspension concentration increased.

- As the storage temperature is decreased from 14 to 4°C, the yeast viability increased and the amount of haze generated decreased. However, yeast growth and metabolic activity were negatively affected as the temperature was decreased from 14 to 4°C. Finally, the amount of protease released decreased as the storage temperature increased. The results show that the yeast cells are more susceptible to damage when cooled at a rapid rate and over a wider range of temperature.

It is evident from this study that exposure of stationary phase *Saccharomyces cerevisiae* to mechanical stress by agitation would lead to loss of yeast quality. It is recommended that the optimum agitation rate should be 400 rpm (impeller tip speed of 1.44 ms^{-1}) using a 1l STR of standard geometry with the yeast suspension diluted to between 30 and 40% wet weight and exposure time should not be greater than 4 hours. In the brewery YCV, the equivalent agitation rate is 48.2 rpm. Future work should focus on using complex impellers such as the helical ribbon to effect mixing as well as the use of bigger reactors of standard geometry (scale-up) in order to be able to compare across reactors with different sizes. Other yeast quality indicators such as the cell surface properties and flocculation and sedimentation characteristics should also be investigated.

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NOMENCLATURE

DEFINITIONS

Attenuation	The conversion of wort sugars to ethanol, CO_2 and other fermentation products leading to a drop in the specific gravity of fermentation media
Attenuation rate	The rate of change in specific gravity of the fermentation medium as a result of the conversion of wort sugars to fermentation products
Consistency	The biomass concentration of yeast suspension expressed as the percentage wet mass.
Cropping	The transfer of yeast from the fermentation vessel to the yeast collection vessel for re-inoculation into subsequent fermentation
Fermentation	The anaerobic process by which yeast cells inoculated into a polysaccharide rich medium (wort) convert sugars to carbon dioxide, ethanol, glycerol and biomass
Flocculation	The agglomeration of yeast during fermentation
Generation number	Indicator of the number of times a batch of yeast has been cropped and re-used
Integrity of the cell envelope	The intactness of the cell envelope
Physiological state	Yeast condition in terms of structural and functional integrity
Pitching	Inoculation of fermentation
Propagation	The step-wise generation of biomass from laboratory to plant scale to obtain sufficient biomass for production purpose
Replicative deactivation	Loss of ability to reproduce
Rheology	The study of the deformation of flow of matter
Sedimentation	The settling of yeast during fermentation

Viability	A term that describes the ability of cells to grow and reproduce; losses of viability may occur via loss of structural integrity, cell death or replicative deactivation
Vitality	A term used to describe the metabolic activity of yeast; in some cases, overall fermentation capacity may be implied
Wort	A cereal-based extract containing a range of carbohydrates which are converted to ethanol, carbon dioxide and other organopletic compounds during beer production
Yeast quality	The physiological condition of the yeast in terms of its fermentative capacity and the quality of the beer produced

SYMBOLS

C	Impeller clearance above the vessel base (mm)
C_f	Final conductivity of tracer (mS/cm)
C_s	Suspension concentration (g/l)
D	Impeller diameter (mm)
F	statistical parameter used in the analysis of variance
h	A function of the osmotic pressure (Pa)
H	Height of liquid in vessel (mm)
h_R	Convective heat transfer coefficient ($W/m^2.K$)
H_u	Length of impeller hub (mm)
K	Consistency index
k	thermal conductivity for the fluid (W/m.K)
k_m	mixing rate constant for turbulent flow ($time^{-1}$)
L	Length of impeller blade (mm)
n	Flow behaviour index
N	Impeller speed (rps)
N_{Fr}	Froude number
N_P	Power number
N_{Pr}	Prandl number
N_{Re}	Reynold's Number
P	Power dissipated by impeller (W)
r	Exponent in Reuss's equation which is a dependent on the yeast morphology
s	standard deviation
s_p	pooled standard deviation

$s_{\bar{x}}$	standard error
S	Substrate concentration ($g.l^{-1}$)
SS	Sum of squares
T	Tank diameter (mm)
T	Temperature (°C)
t	statistical parameter that refers to the deviation from the mean as a means of assessing the statistical significance of the difference between means
t_m	time to achieve uniformity (s).
t_t	t-value obtained from t-tables
U	Fractional degree of uniformity
$V_{control}$	Viability of control (%)
V_{exp}	Viability of actual experiment (%)
W	Width of impeller blade (mm)
$\bar{x}$	Mean or average of a population
X	Biomass concentration ($g.l^{-1}$)

GREEK SYMBOLS

ε	Volume fraction
γ	Shear rate (s^{-1})
μ	Monod specific growth rate (h^{-1})
μ	Viscosity (cP)
μ_{max}	maximum specific growth rate (h^{-1})
ρ	Density (kgm^{-3})
σ	population standard deviation
τ	shear stress ($N.m^{-2}$)

SUBSCRIPTS

66.7, 95%	66.7% and 95% mixing times
Bulk	bulk of fluid
f	final
i	initial
m	mixing time
max	maximum
o	supernatant
s	suspension
Wall	vessel wall
X	Biomass

ABBREVIATIONS

ANOVA	Analysis of variance
Mg-ANS	Magnesium salt of 8-anilino-1-naphthalene sulfonic acid
MYPG	Malt, yeast, peptone and glucose media
PBS	Phosphate buffered saline solution
SAB	South African Breweries
STR	Stirred tank reactor
UCT	University of Cape Town
YCV	Yeast Collection Vessel

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND AND OBJECTIVES OF STUDY

In beer brewing, yeast, mainly of the genus *Saccharomyces*, is used to convert sugars in cereal-based extracts to ethanol, carbon dioxide and organoleptic compounds. The process can be split into three sub processes. These are wort preparation, fermentation and yeast handling, and post-fermentation treatments (Basson, 1996). The yeast-handling sub-process involves the removal of yeast from the fermenter (cropping) for re-use after fermentation is complete. During cropping, yeast is transferred via a heat exchanger, where it is cooled from 14°C to 4°C to a collection vessel. The next stage is storage. In this stage, the cooled yeast is kept in the collection vessel for between 4 to 48 hours after which it is re-used to inoculate the next fermentation, a process known as pitching. During yeast storage, the slurry is agitated to maintain suspension homogeneity and prevent the occurrence of hotspots within the slurry. The vessel is situated in a temperature-controlled room at 8°C. The temperature of the vessel is maintained at 4°C with a cooling jacket. The vessel is operated under anaerobic conditions to hold the yeast in a metabolically inactive state.

As a result of the various stresses the yeast cells are subjected to, their quality and hence performance does deteriorate with time in the brewery. It is reported that the majority of the brewers discard yeast after using it 7 to 10 times (Boughton 1983, Knudsen 1985). At the South African Breweries the yeast is re-pitched 6 to 8 times to minimise poor yeast performance. Possible sources of such stresses could include: agitation, flow through pumps, pipe reductions and valves, exposure to low temperatures, rate of change in temperature, disparities in water potential, ethanol stress, budding cycles and depletion of cellular reserve molecules of yeast in storage (SAB, 1993). Agitation has countering effects on yeast quality: one is to maintain homogeneity and prevent hotspots and on the other hand, subjecting the cells to mechanical stress.

Along with other factors, the quality of yeast is an important determinant of the course of fermentation (Basson, 1996). Improper handling of yeast during the yeast handling process may result in the loss of yeast quality, which would eventually lead to slow, or incomplete fermentations or beer of unacceptable quality. The objective of this study is to investigate the effect of agitation on yeast quality. The yeast collection vessel is replicated on laboratory scale and the slurry subjected to varying agitation rates and dilution factors. Various analytical techniques, which quantify yeast quality, are then used to analyse the agitated yeast.

1.2 THESIS STRUCTURE

A critical review of the literature on the indicators of yeast quality, effect of stresses on yeast quality and rheology of yeast suspension is discussed in Chapter 2. Special emphasis is placed on mechanical stress through agitation and how it affects yeast quality. The link between the rheology of fluids, viscosity of fluids and mixing in stirred tank reactors is also established.

In Chapter 3, the methodology used in determining the effect of agitation on yeast quality is given. The methods by which the yeast rheology was measured and the degree of mixing in the bioreactor was quantified are described. Further, the YCV is compared and contrasted with the experimental rig. A review of the different methods by which yeast quality could be assayed are also given in this chapter.

The relevant statistical tools as well as the principles behind them are described in Chapter 4. The application of these tools to data obtained was shown and the statistical significance of the results established. The reproducibility of the results obtained was determined and the errors and confidence limits for certain assays ascertained.

Chapter 5 deals with the determination of the rheology of the yeast suspensions obtained from the YCV of the Newlands Brewery of SABMiller in Cape Town. The relationship between the apparent viscosities and the concentration of yeast suspension was established by using several mathematical models. The mixing and extent of homogeneity in the experimental rig was quantified and the relationship between mixing time, agitation rates and suspension concentration established. The extent of homogeneity was also determined as a function of the type of impeller in use, impeller speed and suspension concentration.

The results obtained from the investigations described in Chapter 3 are reported in Chapters 6, 7 and 8. In Chapter 6, the results obtained from experiments conducted to look into the effect of intensity and duration of agitation on yeast quality. The intensity of agitation was varied by changing the impeller types and the agitation speed. The experiments were conducted using a laboratory model of the YCV. Chapter 7 contains the results of the experiment conducted to establish the effect of dilution (changing suspension concentration) on indicators of yeast quality. The results obtained from the experiments performed to ascertain the effect of storage temperature on yeast quality are shown in Chapter 8.

In Chapter 9, conclusions drawn based on the findings of the study as well as recommendations for future work are reported. Further, recommendations are put forward with respect to ways by which damage to stationary phase yeast cells can be minimised in stirred tank reactors.

There are four appendices to the text. Appendix A provides the recipe for the reagents used in the different assay methods and Appendix B presents the detailed assay methods. In

Appendix C, the detailed results of the statistical analyses to determine the significance as well as variations of the data are presented. The raw data and history of the yeast suspensions used in this study are shown in Appendix D and Appendix E gives the method by which some of the instruments used were calibrated.

CHAPTER 2: LITERATURE REVIEW

In this chapter, a critical review of the literature on the indicators of yeast quality, effect of stresses on yeast quality and rheology of yeast suspension were discussed. Special emphasis is placed on mechanical stress through agitation and how it affects yeast quality.

2.1 INTRODUCTION

The lager fermentation process at SABMiller involves the use of yeasts to convert sugars to ethanol (SAB, 1993). After fermentation is complete, the *Saccharomyces carlbergensis* settles while *Saccharomyces cerevisiae* flocculates and is stored in the yeast collection vessel (YCV). Yeast storage is often neglected and yet it forms an integral part of yeast management and the final beer quality (Boughton, 1986). The yeast storage and handling area requires very little effort to operate relative to its importance where yeast quality is concerned in maintaining fermentation performance. During storage, the yeast is agitated to maintain homogeneity, kept metabolically inactive by keeping a nitrogen headspace and the surrounding temperature maintained at 4°C. Storing the yeast suspension in the YCV can lead to physiological and mechanical stresses which could have an adverse effect on its quality.

2.2 YEAST QUALITY

The importance of yeast quality cannot be over-emphasised. Final beer quality, taste and flavour of the beer and re-use of yeast are a few of the many factors that are affected by yeast quality. Not only does yeast quality affect beer quality but it also has an overall effect on the economics of the brewing industry. These factors have motivated a study focusing on the effect of stresses on yeast quality (Basson, 1996; Robinson, 2001 and Nkosi, 2001).

To determine yeast quality, one has to analyse the fermentative capacity of the yeast and the quality of beer it produces. The following aspects can be used as indicators of yeast quality:

- The flocculation and sedimentation characteristics of the yeast.
- Yeast viability
- The surface properties of the yeast
- The integrity of the cell envelope
- Yeast vitality

2.2.1 FLOCCULATION AND SEDIMENTATION CHARACTERISTICS

Predicting fermentation performance includes determining the flocculation capacity of the yeast slurry. It is extremely important to the brewing industry to predict the capacity of yeast to consistently flocculate following serial re-pitching (Smart *et al.*, 1995).

Although the mechanism of flocculation is not fully understood, it has been shown that flocculent yeast strains exhibit 'hairy' surfaces whereas non-flocculent strains are smooth (Smart *et al.*, 1995). This 'hairy' surface is made up of tiny glycoprotein filaments, called lectins. Flocculation of brewer's yeast arises from lectin-like interactions where the protein binds to the side chains of α–mannan receptors of adjacent cells. Calcium ions also play an important role in flocculation, as they are specifically required to promote an active conformation of the surface protein. Sugars are effective inhibitors of flocculation, which explains the dispersion of the yeast cells after inoculation into the wort with high sugar content. Flocculation then takes place once the maltose within the wort has been used up.

Flocculation allows for sedimentation to occur thus making it easier for the yeast to be removed from the fermentation vessel. The onset of flocculation and the rate of sedimentation is a balance between the ease of yeast removal, and a sufficient length of time of free yeast suspension so that there is efficient utilisation of wort. Walker (1998) proposed that the occurrence of flocculation has to happen with precision in timing. If it occurs too early, fermentation will cease without all the sugars having being utilised and if too late, the yeast cells will settle out too slowly leading to problems in downstream processing.

2.2.2 YEAST VIABILITY

The attenuation limit of yeast reflects the number of the individual carbohydrates of the wort, which it may ferment. The rate of attenuation is largely dependent on the number of cells in suspension and their ability to metabolise and reproduce (Basson, 1996). Yeast viability refers to the fraction/percentage of yeast cells that are able to metabolise and reproduce in a suspension. Consequently, yeast viability is an important determinant of its quality as sufficient biomass growth after inoculation is required to cause an acceptable attenuation rate. Poor yeast viability results in gradual deterioration in the performance of the yeast in successive fermentations as well as a gradual reduction of yeast crop. The cell membrane is linked to yeast viability in that a non-functional cell membrane leads to replicative deactivation of the yeast cell, which is the first step towards cell death.

The yeast viability can be measured by either quantifying the loss of metabolic activity, loss of replication or looking at cell damage (Jones, 1987 in McCaig, 1990). Loss of replication can be measured by the use of direct plate count and slide culture while, the loss of metabolic activity can be measured by the subjecting the yeast culture to a small-scale fermentation. Finally, cell damage can be measured by the use of a number of dyes such as the methylene blue, eosin-Y and the Mg-ANS stains (King *et al.*, 1981 and McCaig, 1990) to mention a few.

2.2.3 YEAST CELL SURFACE PROPERTIES

Changes in yeast quality can be associated with changes in cell surface properties. Smart *et al.* (1995) concluded that any changes in the cell surface properties would give an indication of the physiological state of the cell. Subjecting the cell to physiological stress could compromise its quality. Cell surface properties can be quantified by measuring the surface charge and hydrophobicity (Smart *et al.*, 1995). Cell surface hydrophobicity is believed to be a function of cell wall component localisation and composition. Hydrophobicity is thought to be determined by the lipid content and the extent of the mannoprotein phosphorylation of the outer cell wall (Smart *et al.*, 1995). *Saccharomyces cerevisiae* cells have negatively charged cell surfaces due to the phosphate groups in their outer cell wall mannoprotein layers. A correlation was found between the ratio of nitrogen to phosphate content and the electrostatic charge. Consequently this ratio is inversely proportional to yeast cell hydrophobicity (Robinson, 2001). The stability of the yeast suspension is determined by the charge being carried by the yeast. Other groups that impart charge on the cells are the carboxyl groups which give a negative charge and the amino groups which are positively charged under the typical pH conditions of the suspensions (Smart *et al.*, 1995).

Amory & Rouxhet (1988) observed that the hydrophobicity of the top fermenting yeast strains is greater than that of bottom fermenting yeast strains while Smart & Whisker (1996) discovered that there are changes in the surface properties during storage and serial repitching. An investigation into the effect of starvation on cell surface charge and hydrophobicity of BB5 yeast strain by Smart *et al.* (1995) indicated a significant reduction in cell surface charge on starvation although the hydrophobicity indices did not change. Some

other work done by Smart & Whisker (1996) on the effect of serial repitching on the hydrophobicity of an ale strain showed that there was no significant change in the cell hydrophobicity irrespective of the number of times the cell was reused. Robinson (2001) investigated the difference between the surface properties of anaerobically and aerobically propagated yeasts and concluded that aerobic propagation yields yeast which is less hydrophobic and more negatively charged.

2.2.4 THE CELL ENVELOPE INTEGRITY

An important aspect of yeast quality is the physical and functional integrity of the cell envelope. The cell envelope is made up of a rigid cell wall and a plasma membrane. The membrane is separated from the cell wall by a periplasmic space (Tuite & Oliver, 1991). The cell wall is so rigid that some of the enzymes secreted by the yeast cells end up getting trapped in the periplasmic space (Tuite & Oliver, 1991). A schematic of the cell wall structure is outlined below in Figure 2.1.

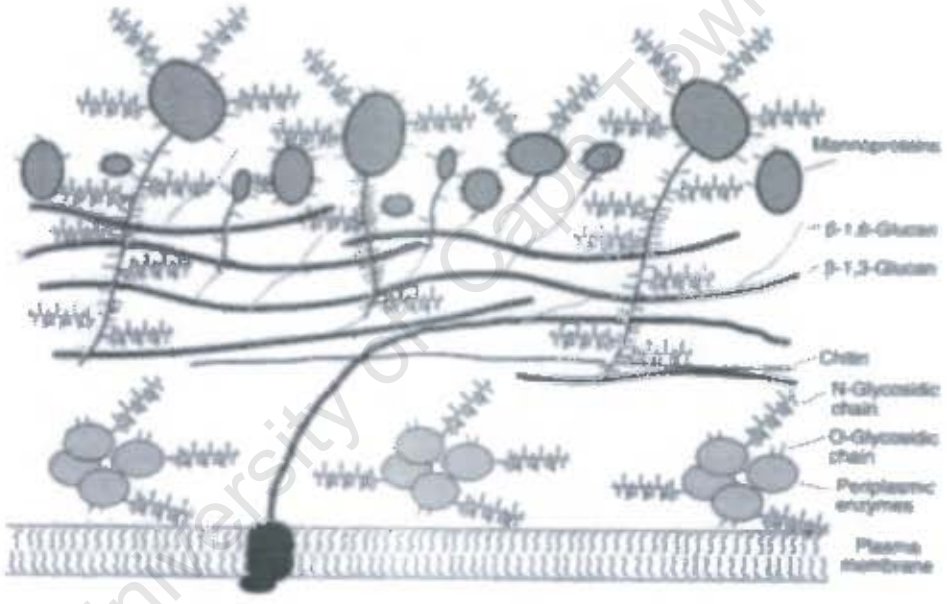


Figure 2.1: Schematic of the yeast cell envelope. Adapted from Walker, (1998)

The cell wall consists of mainly mannoprotein and glucan. There are other cell wall constituents such as chitin and lipid but these are present in relatively small amounts. Glucan is a long twisted chain containing both hydrophilic and hydrophobic sides (Figure 2.2). The hydrophilic side chain contains hydroxyl groups whilst the hydrophobic side contains methane groups. The hydrophobic side chains interact with one another and forms a double helix; it is this double helix form that describes the rigidity of the yeast cell envelope. It has been predicted that the covalent bonds between mannoprotein and glucan and hydrophobic interactions between mannoproteins gives the inner cell wall its structure. In other words, a change in the amount of mannoprotein in the yeast suspension from which yeast has been

removed will give an indication of cell wall integrity (Basson, 1996). An increase in the amount of mannoprotein released indicates increase in cell damage.

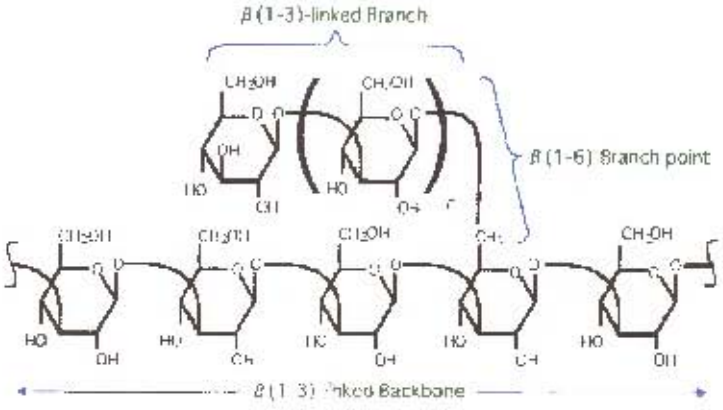


Figure 2.2: Glucan structure. Adapted from Immunity Information Network (2003)

Loss of cell membrane integrity results in the release of intracellular compounds into the beer, which has an effect on its quality (McCaig & Bendiak, 1985b). These compounds affect the flavour characteristics and the clarity of the beer by forming beer hazes (O'Connor-Cox, 1994). The hazes formed can affect the filterability of the beer because of the increase in the concentration of fine particles (Siebert *et al.*, 1987). The formation of beer hazes is associated with the release of glucan, mannoprotein and the enzymes invertase and melibiase from the cell wall (Lewis and Poerwantaro, 1991). Depending on the ratio of protein to carbohydrate in the haze material, the origin of the material can be determined i.e. whether it is from the cell wall, the cell contents or barley (Siebert *et al.*, 1981). This gives an indication of whether the cell envelope disruption is partial or complete. Siebert *et al.* (1981) found that the amount of haze in yeast slurry can be measured by particle size distribution. Other compounds that are released as a result of loss of cell wall integrity are proteases. Release of protease has a negative effect on the foam stability of beer (Ormrod *et al.*, 1991).

2.2.5 YEAST VITALITY

Daoud and Searle (1986) defined vitality is a measure of the physiological state of yeast. It is important that yeast vitality is assessed before the yeast can be used in fermentation. In the brewery, yeast vitality is quantifying by measuring its viability. Daoud and Searle (1986) quantified yeast vitality by measuring the specific oxygen uptake rate and they concluded that there is a direct relationship between fermentation performance and yeast vitality. A change in the physiological state of yeast can result from either a change in the metabolic pathways leading to compromised beer flavours or changes in metabolic rates and biomass growth.

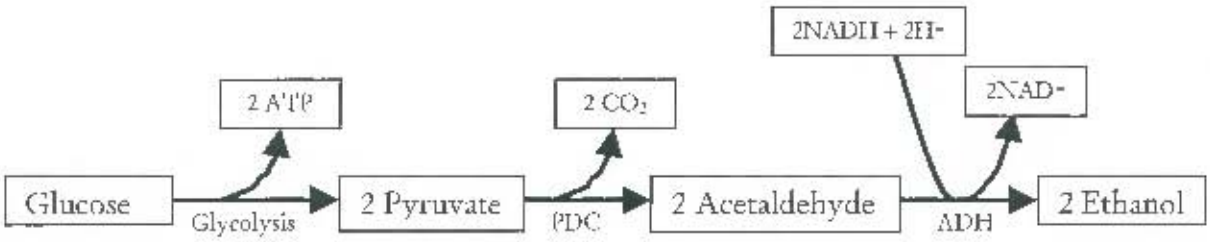


Figure 2.3: Embden-Meyerhof-Parmas pathway. Adapted from Walker (1998)

During fermentation, yeasts convert sugars such as maltose and sucrose firstly to monosaccharides (glucose), then to pyruvic acid and finally to carbon dioxide, energy and ethanol. This metabolic pathway by which glucose is converted to ethanol as shown in Figure 2.3 is known as the Embden-Meyerhof-Parmas (EMP) pathway. Aside from these principal products, other minor products such as fusel alcohols, esters, organic acids and aldehydes are also formed (Walker, 1998). Examples of these minor products that are regularly monitored in beer to assess its flavour profile are: acetaldehyde, sulphur dioxide and diacetyl. Acetaldehyde is formed from the interaction between pyruvate and pyruvate carboxylase, sulphur dioxide is formed from the biosynthesis of cysteine and methionine while, diacetyl is formed from the metabolism of amino acids. These minor products vary depending on the yeast strain and culture conditions and they contribute to the beer flavour (Walker 1998). The presence of these compounds in excessive amounts will cause an undesirable beer flavour. In a study by Pickerell *et al.* (1991), the effect of yeast handling on beer flavour was investigated and it was concluded that adverse yeast handling resulted in high levels of diacetyl and sulphur dioxide at the end of fermentation. High temperature, prolonged yeast storage, bacterial contamination and the cell concentration are some of the factors that affect diacetyl production.

Anaerobic fermentation in the brewing process involves the conversion of maltose to ethanol and carbon dioxide. From the stoichiometric equation of this process, the yield of ethanol on maltose should be 48.8% but it usually is less than that because of the accumulation of storage products such as trehalose and glycogen, the initial aerobic growth of yeasts and ethanol inhibition. During aerobic growth, stored glycogen is metabolised in the presence of oxygen to sterols, unsaturated fatty acids and energy (Pickerell *et al.*, 1991). The energy produced as a result of this metabolism is used for maintenance and growth (Walker, 1998). This implies that the rate of yeast metabolism is affected by initial glycogen concentration and the amount of oxygen present in wort (Pickerell *et al.*, 1991).

2.3 POSSIBLE STRESSES ON YEAST

The yeast cropped and entering the yeast handling circuit is in the stationary phase and in a harsh environment containing minimal nutrients as well as high ethanol concentrations. In storing the yeast suspension, certain conditions such as the exposure to oxygen, the storage temperature and the effect of nutrients and ethanol need to be taken into account. Furthermore, the potential for cumulative stress effects owing to prior stresses generated by flow conditions, cooling rates and yeast history need to be considered. There is a close interrelationship between all the physiological stresses (Walker, 1998). This implies that cells that have been previously stressed by a certain physiological stress may be more sensitive and easily damaged by another physiological stress. Increasing our knowledge on biological response to stress may also allow such responses to be used to build enhanced yeast resilience.

2.3.1 OXIDATIVE STRESS

Oxygen is a chemical that can stress the cells especially during aerobic growth (Walker, 1998). Other effects of oxidative stress include damage to the proteins, lipid and DNA (Walker, 1998). Should a yeast cell be subjected to oxidative stress, certain antioxidant chemicals and enzymes are able to detoxify any active oxygen. An example of such antioxidants is glutathione which destroy oxygen radicals and thus provides a redox balance for yeast cells. Walker (1998) states that a practical way of preventing oxidative stress is to store yeast cells under an inert gas such as nitrogen. There is a close relationship between heat-stress and oxidative stress (Jamieson, 1992 in Walker, 1998). The presence of oxygen leads to an increase in the metabolic activity of cells which subsequently leads to increased temperature and depletion of internal energy reserves such as trehalose and glycogen (O'Connor-Cox, 1994)

2.3.2 TEMPERATURE STRESS

Growth and metabolic activity of yeasts at various temperatures is a function of the genetic background, composition of the medium and other growth parameters (Walker, 1998). Yeast quality will rapidly deteriorate to death and cause autolysis of the cells if it is stored at a high temperature allowing metabolic activity under starvation conditions (Smart *et al*, 1995).

Prior to storage at 4°C, the yeast suspension is cropped and cooled to between 2 and 4°C as quickly as possible. The temperature is not allowed to fall below 0°C as freezing can easily result in cell death owing to ice crystal formation. The purpose of the yeast collection vessel is to store yeast under conditions such that the yeast is inactive and does not undergo physiological stress, which would result in loss of yeast quality. The temperature at which the yeast is stored and its previous cooling history affect the yeast quality. Nkosi (2001) demonstrated that yeast subjected to rapid cooling (2 – 4°C/s) resulted in greater stress with simultaneous damage to the cell envelope (fragility) and loss of yeast vitality as compared to

that cooled slowly (1°C/h). Similar trends were observed for yeast cooled to 4°C over that cooled to 12°C.

2.3.2.1 High Temperature Stresses

Heat affects the cell physiology by disrupting hydrogen bonds and the hydrophobic interactions in the cells. Consequently, the proteins and nucleic acids present in the cells become denatured. Table 2.1 shows some of the effects of high temperature stress on cell physiology.

Table 2.1: Effects of high temperature stress on cell physiology. Adapted from Walker, (1998).

Physiological function	Comments
Cell viability	At the highest growth temperature of many types of yeast, there is appreciable cell death. At supramaximal growth temperatures, thermal death rate is exponential.
General cell morphology	Atypical budding, irregular cell growth and increased cell size
Cell division and growth	Growth of non-thermotolerant yeasts inhibited at temperatures >40°C. Actively dividing cells in S-phase are more thermosensensitive compared with resting cells. Heat shock transiently arrests cells in G1 phase of the cell cycle.
Plasma membrane structure/ function	Increased fluidity and reduced permeability to essential nutrients. Decrease in unsaturated membrane fatty acids Stimulation of ATPase and RAS-adenylate cyclase activity Decline in intracellular pH
Cytoskeletal integrity	Extensive disruption of filaments and microtubular network.
Mitochondrial structure/ function	Decrease in respiratory activity and induction of respiratory-deficient petite mutants Abnormal mitochondrial morphology
Intermediary metabolism	Inhibition of respiration and fermentation above T_{max}. Immediate increase in cell trehalose following heat shock
Protein synthesis	Repression of synthesis of many proteins but specific induction of certain heat-shock proteins. Mitochondrial protein synthesis more thermo labile than cytoplasmic.
Chromosomal structure/ function	Increased frequency of mutation of mitotic crossover and gene conversion. Inefficient repair of heat damaged DNA.

A phenomenon known as thermotolerance has been defined as the temporary ability of cells subjected to high temperatures to survive subsequent lethal exposures to high temperature (Walker, 1998). Understanding the meaning of thermotolerance will help to conveniently explain high temperature stress. Walker (1998), stated that yeast thermotolerance increases to a maximum when the external pH reaches 4.0 while stationary phase yeast are less sensitive to heat and other stresses compared to cells growing rapidly in a glucose-rich medium. When yeasts are exposed to high temperatures, they show a rapid molecular response known as the heat-shock response (Walker, 1998). The proteins that are synthesised as a result of heat-shock response are known as heat-shock proteins (Hsps). Hsps are involved in the degradation of stress-damaged proteins by enhancing the flow of substrates through proteolytic pathways. Hsps are not only synthesised during heat-shock but can also be

induced by ethanol and heavy metal ion stresses (Walker, 1998). Aside from heat-shock proteins, other protective compounds are synthesised as a result of heat shock such as trehalose (Estrusch, 2000). Iwahashi *et al.* (1995) reported that trehalose acts as a thermoprotectant by increasing the thermal stability of yeast cellular proteins and stabilising the cell membranes. Temperature stability by trehalose is attained by the formation of a hydration protection layer around the proteins.

2.3.2.2 Low Temperature Stresses

The mechanism by which yeasts cells are stressed as a result of low temperature stress is still unclear (Walker, 1998). However, low temperature stress causes yeast cells to shrink uniformly and an increase in the polyunsaturated membrane fatty acids. The increase in membrane fatty acids leads to a decline in solute transport. Walker (1998) also stated that the low temperature stress leads to formation of leaky membranes, vacuolar damage and consequently splitting of vacuoles. Not only is the cell morphology and integrity affected, its fermentative performance is also compromised and expression of specific proteins induced. Fargher and Smith (1995) showed that applying low temperature stress to brewing yeast strains prevents budding and leads to the re-arrangement of the vacuoles. The yeast strain in the study by Fargher and Smith (1995) was exposed to cold shock at 4°C. Komatsu *et al.* (1990) used *Saccharomyces cerevisiae* cells that had been exposed to hear shock at 43°C for 30 min before freezing in liquid nitrogen and thawing to prove that heat shock increases cell viability. The rationale for this is that the hsps induced during the heat shock protected the cells by increasing hydrophobic interactions within the cells and forming stable macromolecules. McCaig and Bendiak, (1985b) investigated the effect temperature of storage on yeast samples under beer for 6 days. The storage temperatures used in this investigation are 1, 5, 10, 15, 20 & 25°C. They concluded that pitching yeast stored at temperatures above 5°C, perform poorly in subsequent fermentations by having a longer lag phase, poor rate of attenuation, higher final pH values and lower alcohol yields. The rationale given by McCaig and Bendiak, (1985b) for this poor performance is that the elevated temperatures accelerate the metabolism of the yeast cell resulting in the consumption of stored internal reserves.

2.3.3 NUTRIENT STRESS

The presence of sugar substrate and nutrients such as amino acids at moderate temperatures promotes cell growth. The lack of nutrients can result in cell starvation. Studies carried out investigating the effect of nutrient starvation showed that it affected yeast quality (Smart *et al.*, 1995). It was observed that starved cells were less flocculent in beer and that the cell surface topography was changed. The cell surface charge was also affected as starved cells were found to be less negatively charged when compared to non-starved cells. The only surface property that was not affected by starvation was hydrophobicity. When yeast cells are starved, the amount of stored internal reserves such as trehalose is increased (D'Amore, 1991). The function of trehalose in this case is to act as a protectant against nutrient stress.

2.3.4 MECHANICAL STRESSES

Fluid forces exerted on the yeast cells in the YCV are affected by the:

- rate of agitation (impeller speed)
- apparent viscosity of the fluid
- bubble disengagement
- impeller design and
- tank geometry.

In Table 2.2, a summary of the factors determining the sensitivity of microorganisms to shear is given. Under typical agitation conditions, cells encounter two types of shear forces. Due to fluctuating velocity components caused by turbulence close to the impellers, the cells encounter fluctuating forces (Reynolds stresses). However, in the bulk of the reactor, they are exposed to laminar shear forces due to gradients in the flow (Metzner and Otto, 1957).

Table 2.2: Factors affecting shear sensitivity. Adapted from Chisti (1999)

1. Type of cell and species
2. Composition and thickness of cell wall when present
3. Size and morphology of cell
4. The intensity and nature of shear stress.
5. Growth history (both short-term and long-term adaptation), environment, rate and stage
6. Type and concentration of shear-protective agents if present

Dunlop *et al.* (1994) carried out a study where the sensitivity of a plant cell culture to fluid forces in a stirred bioreactor was assessed by shearing the cells under both laminar and turbulent conditions. The plant cells used in the study by Dunlop *et al.* (1994) were *Daucus carota, Petunia Mitchell* and *Glyceine soya* grown in MS basal media. The biological parameters measured were re-growth ability, mitochondrial activity, aggregate size and lysis. The shear stresses were increased from 0.5 to 10kPa under laminar conditions and imposed for an exposure time of between 1 and 60 minutes. The viability of the cells was completely destroyed at a shear stress of 0.1kPa and an exposure time of 10 minutes, while the membrane integrity was not affected. However, a shear stress of 10kPa and an exposure time of 60 minutes damaged the plasma membrane significantly and caused cell death.

The turbulent conditions were attained by using impeller speeds ranging from 32 to 1000rpm and exposure time from 1 to 240 min. Under turbulent conditions at low agitation speeds of 32 – 512 rpm and an exposure time of 4 hours, the mean aggregate size was not affected but increasing the speed to 1000 rpm and an exposure time of 60 minutes led to a decrease in the mean aggregate size. These results show that fluid-mechanical sensitivities are highly dependent on the intensity of the forces. Increased fluid shear forces are therefore able to cause damage to organelles (mitochondria), reduce cell viability, destroy the cell membrane, reduce flocculence (mean aggregate size) and cause cell death (lysis).

Generally yeast cells are quite robust and not easily damaged by mechanical stress because of the thickness of their cell walls (Walker, 1998). The resilience of yeast cells is emphasised by the severity of the mechanical stress needed to rupture the cells in order to extract cell components.

In a study by Lamaignére (2002), growing yeast cells (*Saccharomyces cerevisiae*) were subjected to agitation in a stirred tank slurry bioreactor in the presence of inert particles (0 – 5% volume fraction). A Rushton turbine was used at impeller speeds of between 460 and 850 rpm (impeller tip speeds of 1.92 – 3.52 ms^{-1}). It was discovered that viability and the metabolic activity of the cells decreased at impeller tip speeds exceeding 2.48 ms^{-1} and that the cell damage became more pronounced if the volume fraction of inert particles is increased. Finally, there was severe cell envelope damage at high agitation rates. Consequently, Lamaignére (2002) concluded that the most favourable agitation rate for yeast growth is at an impeller tip speed of 2.33 ms^{-1} in the presence of 1% by volume of inert particles.

2.3.5 ETHANOL STRESS

Ethanol is one of the products of the fermentation process, however, this chemical can also be inhibitory to the cells if present in a high concentration. During cell growth, ethanol acts as a non-competitive inhibitor at low concentrations (Walker, 1998). D'Amore *et al.*, (1990) observed that as fermentation progresses, the intracellular ethanol concentration increases but since ethanol diffuses quickly across the cell membrane, it is not accumulated in the cells. Ethanol affects the cell viability and growth by inhibiting cell division (Walker, 1998). Ethanol toxicity can lead to the induction of heat shock-like proteins and increase the oxygen radicals which enhance cell ageing. Subsequently, the oxygen radicals increase membrane fluidity and compromise membrane structural integrity (Walker, 1998). The inhibitory effect of ethanol is enhanced by a number of factors such as high temperature, nutrient limitations such as Mg^{2+} (Birch and Walker, 2000) and other metabolic products. Yeast cells adapt to ethanol stress by altering their membrane fluidity, increasing the catabolism of ethanol, biosynthesis of stress proteins and increase membrane unsaturated fatty acids (Walker, 1998). Unsaturated fatty acids have the advantage of being ethanol tolerant (Mizoguchi and Hara, 1997). Walker (1998) suggested that the stress proteins act in a damage-repairing role rather than a protective role. Just like in the case of temperature stress, there is an elevated level of cellular trehalose observed in ethanol stressed *Saccharomyces cerevisiae* (Walker, 1998). Osmotic pressure, media composition, modes of substrate by-product formation are some of the factors that dictate ethanol tolerance in yeast cells (Walker, 1998). Ethanol stress is possible in the YCV if the yeast had not been washed or diluted to reduce the residual ethanol concentration before it enters the YCV. In a study conducted by Birch and Walker (2000) on the influence of Mg^{2+} on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*, they found that Mg^{2+} enhanced yeast cultures were able to withstand lethal heat and ethanol stresses and maintain high viabilities.

2.4 RHEOLOGY OF THE YEAST SLURRY

The temperature and duration of storage are factors that need to be carefully controlled to ensure that the quality of yeast is not compromised (McCaig and Bendiak, 1985a, Knudsen, 1985). However, the rate of cooling in a vessel is dependent on a number of parameters such as the yeast rheology, the method by which cooling is effected, the presence of agitation, the shape and geometry of the vessel. The yeast suspension is a fluid of great complexity, as it comprises of three phases (Lenoël *et al.*, 1987):

- the liquid phase, which is the beer.
- the solid phase comprising of the yeast cells
- the gas phase which is usually carbon dioxide.

The rheology of the yeast suspension is highly dependent on the suspension concentration and it can exhibit both Newtonian and non-Newtonian rheology. Its complex nature results in a viscosity which is dependent on yeast concentration, floc size distribution, shear rate, duration of exposure to shear, temperature, pH, osmotic pressure and gas phase present (Reuss *et al*, 1979). In order to select and size equipment and to gain an understanding for the forces that are experienced by the yeast cells, one has to have an appreciation of the suspension rheology.

2.4.1 VISCOSITY OF SUSPENSIONS

Apparent viscosity is a function of the shear rate and the zone (in the vessel) under consideration. The shear rate can be predicted by the impeller speed; therefore it is very important to be able to predict the viscosity of the yeast at different shear rates. The Newton's Law of viscosity shown below describes the relationship between apparent viscosity, μ and shear rate, γ.
Firstly,

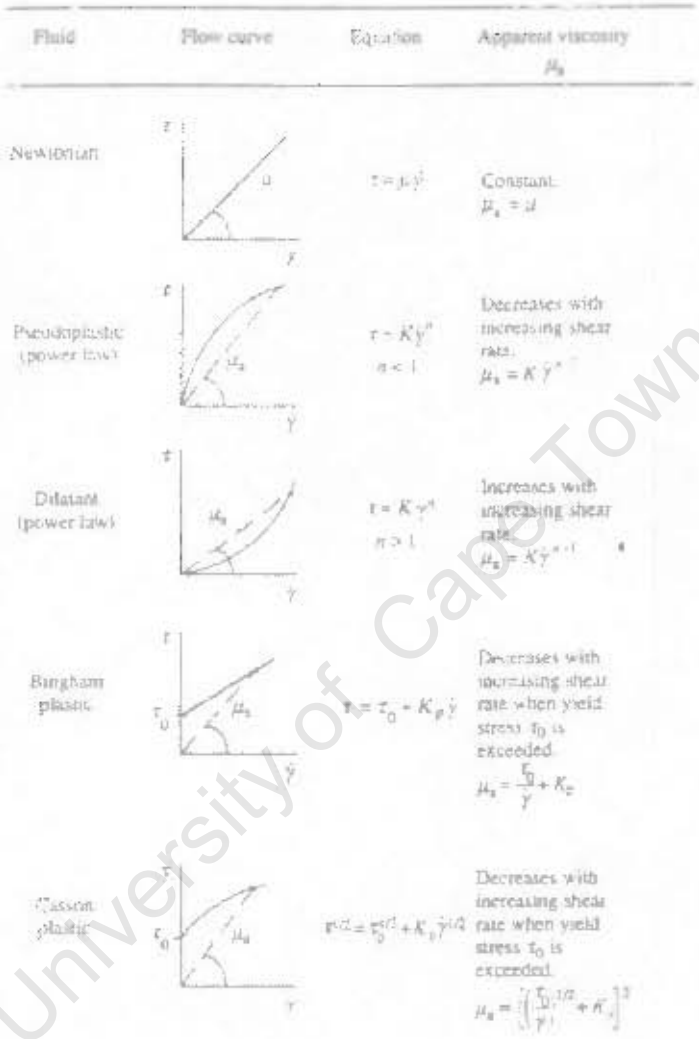
$$\mu = -\frac{\tau}{\dot{\gamma}} \qquad \text{Equation 2.1}$$

where: τ = shear stress (N/m^2)
γ = shear rate (/s)
μ = apparent viscosity (Pa.s)

Fluids have been classified depending on their relationship between the shear stresses and viscosities. Newtonian fluids exhibit viscosities that are independent of the shear rate. However, non-Newtonian fluids have viscosities that are dependent on the shear rate. Shear dependent non-Newtonian fluids have been classified into Bingham plastics, pseudo-plastics, dilatant and Casson plastic. Pseudo-plastic fluids have a viscosity that decreases as the shear rate increases making them shear thinning in nature (Table 2.3). However, the viscosity of

dilatant fluids increases with increase shear stress or rate. Most biological fluids are pseudo-plastic in nature (Doran, 1995).

Table 2.3: Classification of fluids based on their rheology. Adapted from Doran (1995)



Newtonian, pseudoplastic and dilatant fluids are grouped as power law fluids. The rheological equation of state for any power law liquid is given by:

$$\mu_a = \frac{\tau}{\dot{\gamma}} = K\dot{\gamma}^{n-1} \text{ ---------- Equation 2.2}$$

where: K = consistency index
n = flow behaviour index

In the case of pseudo-plastics or shear thinning fluids, n<1 and the apparent viscosity decreases with increasing shear rates. However, the reverse is the case for dilatants or shear thickening fluids. For Newtonian fluids, n is unity. Bingham plastic fluids are fluids that have

to overcome a certain shear stress (τ_o) before any motion is produced. These fluids will then behave like a Newtonian fluid once the yield stress is exceeded. Equation 2.3 shows the equation describing the relationship between the shear stress and shear rate for Bingham plastic fluids.

$$\tau = \tau_o + K\dot{\gamma} \text{ ----------} \qquad \text{Equation 2.3}$$

Similarly, Casson and certain pseudoplastic fluids need to overcome a certain shear stress prior to motion. Beyond this, they exhibit shear-thinning behaviour. The Herschel-Bulkley equation describes the rheology of these pseudoplastics (Equation 2.4) while Equation 2.5 describes the rheology of Casson fluids.

$$\tau = \tau_o + K\dot{\gamma}^{n-1} \text{ ----------} \qquad \text{Equation 2.4}$$

$$\tau^{1/2} = \tau_o^{1/2} + K\dot{\gamma}^{1/2} \text{ ----------} \qquad \text{Equation 2.5}$$

Apart from being shear-dependent, fluids can also be time-dependent. Examples of such fluids are rheopectic fluids in which apparent viscosity increases with time and thixotropic fluids whose apparent viscosity decreases with time. Examples of cultures that exhibit thixotropic behaviour are fungal mycelia and extracellular microbial polysaccharides (Doran, 1995).

The shear rate can be expressed as a function of n and k_i (Chisti, 1999):

$$\gamma_{av} = k_i\left(\frac{4n}{3n+1}\right)^{n/n-1} \times N \text{ ----------} \qquad \text{Equation 2.6}$$

where k_i is dependent on the impeller type used to effect mixing and N is the impeller speed. The k_i values for different impellers are shown in Table 2.4.

Table 2.4: The k_i values for different impellers. Adapted from Chisti (1999)

Impeller	k_i
Six-bladed disc turbine	11 – 13
Paddle impellers	10 – 13
Propellers	~10
Helical ribbon impellers	~30

The shear stress and apparent viscosity at different shear rates can be measured by the use of a viscometer and the values of the flow index of the fluid can be applied to any stirred-tank.

2.4.2 VISCOSITY OF YEAST SUSPENSIONS

Yeast suspension exhibits a pseudo-plastic rheology at dry matter content greater than 40% pressed yeast (as estimated by the Buchner test) and thinner yeast suspensions have a Newtonian rheology (Lenoël *et al*, 1987). Rudiš *et al* (1976) investigated the relationship between the suspension concentration of yeast (*Saccharomyces cerevisiae*) and viscosity in the range of 0 to 10% wet weight and they concluded that the rheology of yeast suspension is Newtonian within that range. Furthermore, they suggested an empirical formula that relates viscosity to suspension concentration and temperature. This formula is given in Equation 2.7.

$$\mu = 1.6758 - 0.02816T + 3.27*10^{-6}C_s^3 - 3.128*10^{-8}C_s^3T \quad \text{——} \quad \text{Equation 2.7}$$

where: μ = viscosity (mPa.s)
T = temperature (°C)
C_s = suspension concentration (g/l)

Other equations that have been shown to describe the relationship between the viscosity of yeast suspensions and its concentration are the Vand and Einstein equations (Reuss *et al.*, 1979). The Einstein equation (Equation 2.8) is only valid for suspensions with volume fractions less than 4% while the Vand equation (Equation 2.9) is applicable to suspensions of higher volume fractions. Since the volume fraction of cells in the YCV is much greater than 4%, Einstein's equation is not applicable.

$$\mu_s = \mu_o\left(1 + 2.5\varepsilon_X\right) \quad \text{——} \quad \text{Equation 2.8}$$

$$\mu_s = \mu_o\left(1 + 2.5\varepsilon_X + 7.25\varepsilon_X^2\right) \quad \text{——} \quad \text{Equation 2.9}$$

Reuss *et al.* (1979) proposed a hyperbolic relationship between the volume fraction of the suspension, osmotic pressure, suspension viscosity and the viscosity of the supernatant (Equation 2.10).

$$\mu_s = \frac{\mu_o}{1 - \left(h_s\varepsilon_X\right)^r} \quad \text{——} \quad \text{Equation 2.10}$$

where: h_s is a function of the osmotic pressure
r depends on the morphology of the yeast.

In the study by Reuss *et al.* (1979), the osmotic pressure of the yeast suspension was varied by suspending the yeasts first in NaCl solution and later in arabinose solution. Since the ionic concentration of the yeast suspension in the YCV rarely varies, the equation obtained by Reuss *et al.* (1979) is not applicable.

2.5 AGITATION

The importance of mixing in the chemical industry cannot be overemphasized. In industry, agitation is provided in a tank for the following purposes: to blend liquids, suspend solids, maintain homogeneity, ensure mass and heat transfer and disperse gas or vapour. Agitation is even more important in biochemical industries where the microorganisms need good oxygen dispersion and temperature distribution to maintain viability and vitality. In the yeast collection vessel, the purpose of agitation is to maintain homogeneity, suspend solids and ensure heat transfer.

Following a rigorous search of the literature the limited amount of information available on mixing in bioreactors is noted. This limitation is most likely due to the complex rheology of fermentation broths. For example, the yeast collection vessel has three states present: solid, liquid and gas. As mixing commences, the amount of gas in the slurry changes and, consequently, so does its apparent viscosity. The yeast collection vessels are operated in the laminar region, because of the high viscosity of the yeast slurry. Diluting the yeast cream from approximately 60% down to 40%, 30% and 20% solids (on a wet basis) is accompanied by concomitant changes in apparent viscosity with concentration.

Bryant (1977) postulates that the more difficult aim to achieve when mixing is the reduction of temperature gradient i.e. enhancement of heat transfer rates rather than a reduction in concentration gradient. One question posed by Bryant (1977) is whether fermenters or bioreactors should be designed in such a way that every microorganism experiences the same environment or should these variations be recognised or accepted in the design. On a small-scale, the spatial variation could be ignored but with scale up of the reactor, these variations become magnified.

In a study conducted by Lewis and Poerwantaro (1991) on the agitation of yeast suspension, it was discovered that agitation results in the release of haze material. The yeast strain used in this case was a brewer's strain of *Saccharomyces cerevisiae* with a dry weight concentration of 10 g/l (~4% wet weight) which was agitated at 300 rpm by the use of a mechanical shaker at 20°C for 5 min. They concluded that the amount of haze released is independent of the temperature of agitation (10 and 30°C) and intensity of agitation (200 and 300 rpm) but dependent on the duration of agitation and pH of the medium. They also observed that cells in the stationary phase were more susceptible to haze release rather than growing cells. Since the yeast cells in question are in the stationary phase, this implies that they are susceptible to cell wall damage and subsequently, haze release.

The extent of agitation in a tank is however dependent on the type of impeller, impeller dimensions with respect to tank geometry, impeller speed and subsequently the amount of power drawn. The use of baffles and residence time are also factors to be taken into consideration. Each of these factors is discussed in detail below.

2.5.1 IMPELLER TYPE AND FLOW PATTERN

The types of impellers available can be classified based on the flow pattern they produce and the range viscosities of the fluid to be mixed (Zlokarnik and Strasse, 2002). Classifying impellers based on flow patterns, there are three categories: radial-flow, axial-flow and close-clearance impellers (Perry *et al.*, 1998). Radial flow impellers project fluid radially (as the name implies) towards the wall of the vessel (Figure 2.4). Their blades are parallel to the axis of the shaft and they may either be curved or flat bladed (Perry *et al.*, 1998). Examples of such impellers are turbines and paddles. Axial flow impellers on the other hand pump fluid upward or downward and each blade forms an angle of less than 90° with the plane of rotation (Figure 2.5). Examples are propellers or hydrofoils and pitched blade turbines (Perry *et al.*, 1998). Finally, close-clearance impellers operate close to the tank wall and are well adapted to viscous fluids (Perry *et al.*, 1998). Examples are anchor and helical impellers. Classifying impellers based on the range of viscosities of fluids they can effectively mix, there are three categories: low, medium and high viscosity impellers (Zlokarnik and Strasse, 2002). Low-viscosity impellers are turbines and propellers and they are useful for mixing fluids with viscosities less than 500 cP while medium-viscosity impellers such as the blade and grid impellers are useful for mixing fluids with viscosities between 500 and 5000 cP. Anchor and helical ribbon impellers are high-viscosity impellers and suitable for fluids with viscosities greater than 5000 cP.

Of all the impeller types mentioned above, the close-clearance flow impellers provide a better mixing in highly viscous fluids (Zlokarnik and Strasse, 2002). It must be noted however that these are likely to cause the most damage to the yeast cells because of the high shear environment that they offer. Of the radial and axial flow impellers, the turbines give a higher shear environment for the fluid to enhance heat and mass transfer and provide better circulation (Coulson *et al.*, 1999). The axial flow impellers are used in cases where low shear and good homogeneity in low viscosity fluids are required (Coulson *et al.*, 1999). Pitched blade turbine is used for high axial circulation and it provides a lower shear environment than the radial flow impeller. The axial flow impellers also draw the less power than the radial flow impellers (McCabe *et al.*, 2001).

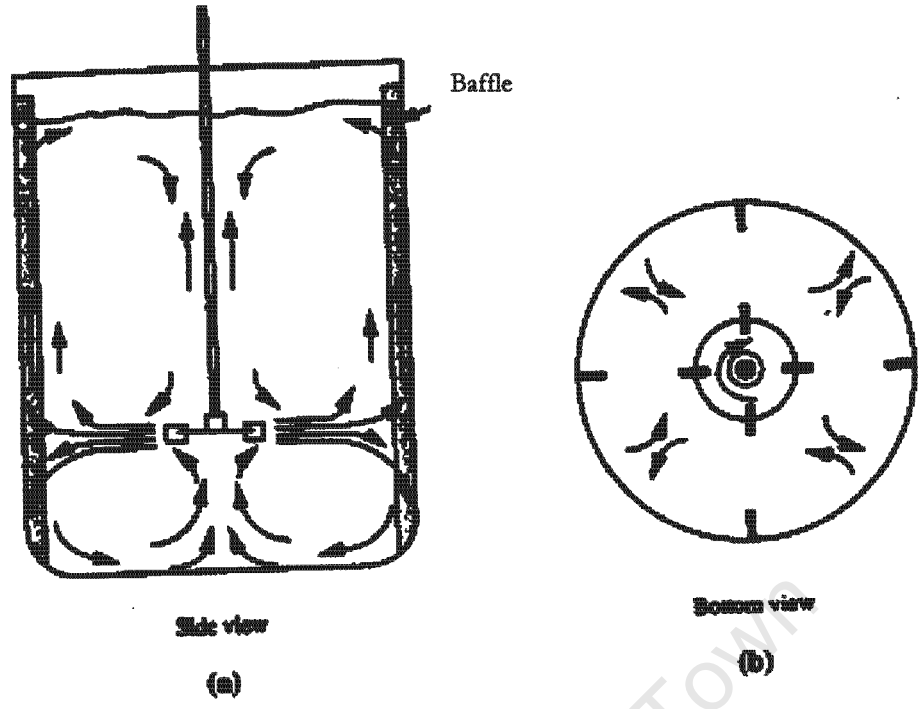


Figure 2.4: Flow pattern produced by Rushton turbine in a baffled tank. Adapted from Rushton *et al.* (1950a)

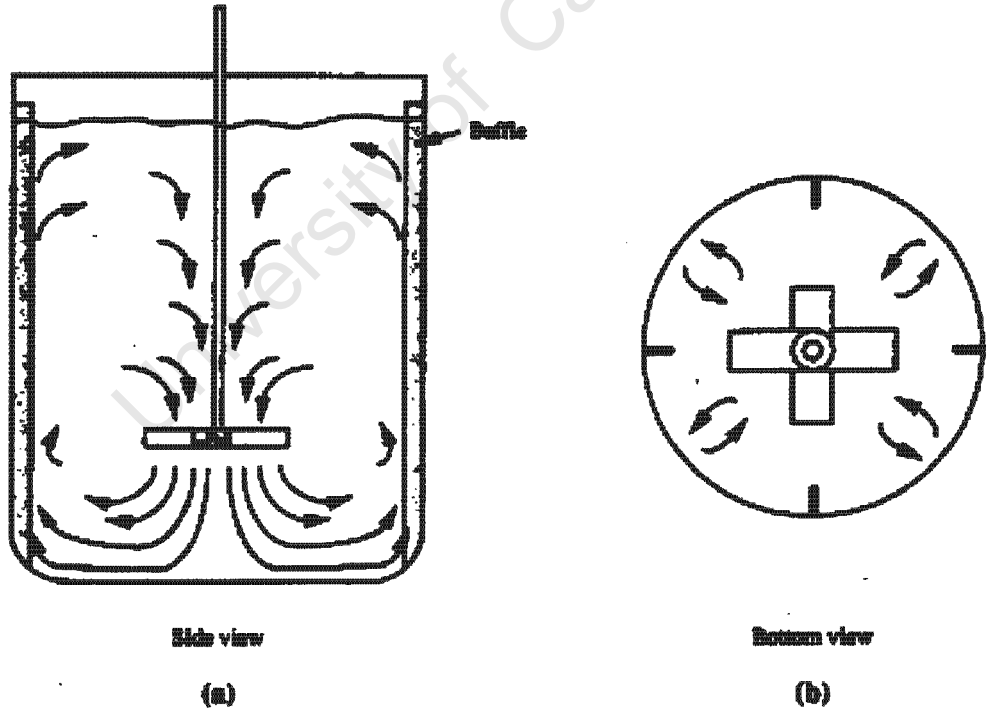


Figure 2.5: Flow pattern produced by a pitched blade impeller in a baffled tank. Adapted from Rushton *et al.* (1950a)

2.5.2 IMPELLER SPEED AND FLOW CHARACTERISTICS

The higher the impeller speed, the more turbulent the flow becomes. The extent of turbulence is shown by the value of the Reynolds number, N_{Re} where:

$$N_{Re} = \frac{D^2 N \rho}{\mu} \qquad \text{Equation 2.11}$$

where:
- D = impeller diameter (m)
- N = impeller speed (rps)
- ρ = fluid density (kg m^{-3})
- μ = fluid viscosity (Pa.s)

The Reynolds number is proportional to the impeller speed hence in the absence of other variations, the higher the impeller speed, the higher the Reynolds number and the closer the fluid motion is toward turbulence. In a mixing tank, flow is in the turbulent region when $N_{Re} > 1000$ (Robinson, 2001) and in the laminar region when $N_{Re} < 10$ (Doran *et al.*, 1998). Transition region is the intermediate region between the turbulent and laminar regions. Flow is in the transition region when flow is turbulent near the impellers but laminar in other regions in the tank (Perry *et al.*, 1998). Further, for pseudoplastic fluids, flow is in the transition region when Reynolds number is about 40 (McCabe *et al.* 2001).

Power consumed by an impeller is a function of the impeller type, size, speed, the physical properties of the fluid, aeration rate and vessel geometry (Oldshue, 1983). In the laminar region, the power requirement is directly proportional to viscosity meaning that mixing of highly viscous Newtonian suspensions have high power requirements. However, in the turbulent region, power requirement is independent of viscosity but is a direct function of fluid density. The Reynolds number may be correlated with the power number (for vessels of similar geometry). In the study done by Rushton *et al.* (1950b) on the effect of several geometrical parameters on power requirements, it was discovered that both the impeller diameter to tank diameter ratio and the fluid depth to tank diameter ratio for turbines did not have an effect on the power requirements However, increasing the impeller clearance to impeller diameter ratio increases the power requirement for a disk turbine and decreases the power requirement for a pitched blade impeller. According to McCabe *et al.* (2001), the shape of the tank has little effect on the amount of power drawn by the impellers. However, the shape of the tank affects the circulation patterns and thus the mixing time.

The power number, N_P is calculated as follows:

$$N_P = \frac{P}{\rho N^3 D^5} \qquad \text{Equation 2.12}$$

where:
- N_P = Power number
- P = Power dissipated by impeller (W)

In comparing the power requirements in mixing a Newtonian fluid and a pseudoplastic fluid, it can be concluded that pseudoplastic fluids consume less power in the range of Reynolds number between 10 and 100 (McCabe *et al.* 2001). However, there is no difference in the power requirements of both systems in the laminar and turbulent regions. In the laminar region, viscosity is a determining factor of power requirements meaning that operating the YCV when the suspension concentration is high (> 60%) will lead to a system that has high power requirements. However, in the turbulent region, the suspension density will be very important.

2.5.3 DIMENSIONS

The dimensions of the impeller are also a determining factor as to the extent of agitation. Impellers having a diameter greater than half the tank diameter are good at avoiding stagnant regions in highly viscous fluids and also provide improved mixing times allowing the enhancement of homogeneity throughout the tank (Perry *et al.*, 1998). A high impeller-to-tank diameter also helps to promote heat transfer because they provide a larger surface area for heat transfer (Blanch and Clark, 1997). Small-diameter impellers also have their uses (these are impellers with diameters less than a third of the tank diameter). They are useful for promoting mass transfer, gas dispersion in slurries and rapid mixing of dry particles into liquid. Thus, the reason for operating a standard mixing tank at an impeller diameter-to-tank diameter ratio of 1/3. The higher the ratio of the impeller diameter-to-tank diameter, the greater the shear stresses in the tank because the stagnant regions near the tank walls are now exposed to agitation.

2.5.4 BAFFLES

The presence of baffles helps to increase the intensity of agitation. The agitation in a baffled tank is more vigorous than that of an unbaffled one irrespective of the fluid viscosity. The vortex that is experienced in unbaffled tanks is eliminated by the use of baffles in large tanks and by mounting the impeller off centre (McCabe *et al.*, 2001). Baffles are not needed for fluids with viscosities less than 10 000 cP (McCabe *et al.*, 2001). Rushton *et al* (1950b) quantified the value of the Reynolds number at the inception of vortex as 300. The fluid forces will be balanced as the presence of baffles also produce some shear force at the walls of the vessel. Since baffles intensify agitation and consequently shear stress, it is expected that the presence of baffles would lead to even further deterioration of yeast quality on agitation as well as reduced vortex due to the rheology of the yeast. Vortex reduction provides a positive effect since it reduces gas entrainment. Baffles generally change the mixing pattern in a tank. It must be noted however that in laminar region, the same amount of power is consumed whether baffles are present or not (Rushton *et al*, 1950).

2.5.5 HEAT TRANSFER

The fluid mechanics of the film at the mixer side of the tank defines the heat transfer at the surface to a greater extent than the behaviour at the impeller zone (Perry *et al*, 1998). The heat can be transferred to a vessel in a number of ways, which include using: jacketed tanks, helical coils, heating coils that act as baffles or even heat exchangers. The helical coils can either be outside or inside the vessel. One of the major advantages of internal helical coils is that they present a larger surface area for heat transfer (Blanch and Clark, 1997). The major consideration of the heat transfer obtained is the fluid flow achieved. The complex impeller usually gives more flow for given power levels than that the axial or radial impellers, resulting in better heat transfer. This is true to a limited degree for jacketed tanks but with internal coils, the type of flow exhibited makes the first and second turn in the coil blank off flow from the turns above it (Perry *et al*, 1998). As a result, the increased flow from this impeller does not aid heat transfer. The heat transfer coefficient will give an indication as to the resistance being encountered in a stirred tank and this can be calculated from:

$$\frac{h_R T}{k} = 0.35\, N_{\mathrm{Re}}^{0.59} N_{\mathrm{Pr}}^{0.33} \left(\frac{\mu_{wall}}{\mu_{bulk}}\right)^{-0.14} N_{Fr}^{-0.1} \qquad \text{Equation 2.13}$$

where:

h_R	=	convective heat transfer coefficient (W/m^2.K)
T	=	tank diameter (m)
k	=	thermal conductivity for the fluid (W/m.K)
μ_{wall}	=	fluid viscosity at vessel wall (Pa.s)
μ_{bulk}	=	fluid viscosity in the bulk (Pa.s)
N_{Re}	=	Reynolds number for the tank (Equation 2.6)
N_{Pr}	=	Prandl number = $\frac{c_p \mu}{k}$
N_{Fr}	=	Froude number = $\frac{DN^2}{g}$

The specific heat capacity of the yeast slurry is approximated to be equal to that of water. This is because yeast biomass consists of 70% water (Walker, 1998). In a case of a highly concentrated suspension with a complex rheology, the heat transfer is influenced by the apparent viscosity near the vessel walls rather than the bulk viscosity (Blanch and Clark, 1997). Lenoël *et al* (1987) suggest that the time taken for the fluid in the middle of the vessel to reach the temperature at the vessel walls (in the case of jacketed vessels) is proportional to the square of the diameter of the vessel. This implies that the bigger the vessel, the longer time it takes to reach homogenous temperature. Calculating the heat transfer coefficient is very important in determining the effectiveness of the heat transfer in the YCV and experimental rig and consequently predicting the source of damage in these vessels.

2.5.6 MIXING TIME

Mixing time is defined as the time taken for the concentration of a material to be blended in a vessel to reach a degree of uniformity following addition of a perturbation such as addition of a tracer. It has been defined for a uniformity of 66.7, 95, 99 and 99.99% depending on what is most suitable for the process. Khang and Levenspiel (1976) discovered that the concentration of the material to be mixed behaved like a sinusoidal damped response with the mixing time curve falling between a pair of exponential curves which are oppositely signed (Figure 2.6).

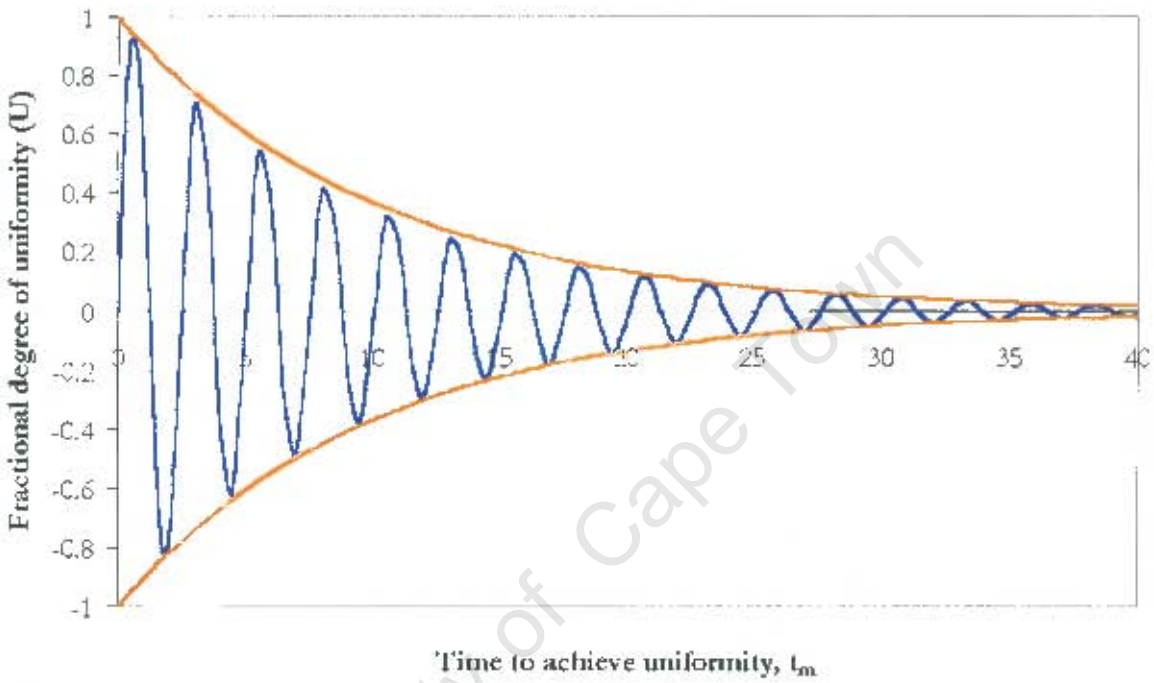


Figure 2.6: Graphical representation of the tracer response

In the case where the dispersion of the tracer is a sinusoidal damped response, Equation 2.14 can be used to calculate the mixing time.

$$t_m = -\frac{\ln(1-U)}{k_m} \quad \text{..... Equation 2.14}$$

where:

U	=	fractional degree of uniformity
t_m	=	time to achieve the uniformity.
k_m	=	mixing rate constant for turbulent flow ($time^{-1}$)

For some industries a mixing time of 90% uniformity is perfectly suitable and the implication of having more or less uniformity has to be determined by careful experimental analysis of the

problem (Fasano and Penney, 1991). If N_{Re} exceeds 5,000 as stated in the introduction, the mixing time is independent of N_{Re}

$$\frac{k_m}{N} = f\left(N_{Re}, symmetry\right) \text{ ——————} \quad \text{Equation 2.15}$$

$$\Rightarrow k_m \alpha N\left(\frac{D}{T}\right)^b\left(\frac{T}{H}\right)^{0.5}$$

$$k_m = a \times N\left(\frac{D}{T}\right)^b\left(\frac{T}{H}\right)^{0.5} \text{ ——————} \quad \text{Equation 2.16}$$

Constants (a and b) for turbines are given in Table 2.5 below. These constants have been calculated when the material was injected from the top surface with axial impellers pumping downwards. Adding the reagents below the axial flow impellers causes a decrease in the mixing rate constant and subsequently, an increased mixing time (Fasano and Penney, 1991). These mixing rate constants shown in Table 2.5 are only suitable for use in a Newtonian system. To compare mixing time of different impellers, impellers should be loaded to the same motor, gear, reducer, mounting adaptor, seal and shaft. For very low N_{Re}, streamline flow occurs and the mixing time will be prohibitively long.

Table 2.5: Mixing rate constants. Adapted from Fasano and Penney (1991)

Impeller Type	a	b
Six-bladed Disk Turbine	1.06	2.17
Four-Bladed Flat Turbine	1.01	2.30
Four-Bladed Pitched Turbine	0.641	2.19
Chemineer HE-3 Turbine	0.272	1.67
Square Pitch Marine Impeller	0.274	1.73

Another parameter that can be measured when the degree of homogeneity in a vessel is to be characterised is the circulation time. This is a function of the pumping capacity of the impeller and it is the time taken for a molecule of fluid to complete a closed loop (Guérin *et al.*, 1984). Comparing the axial and radial-flow impellers in geometrically similar vessels, the circulation time is much shorter for the radial-flow impeller as the fluid element travels through a shorter loop (Figure 2.4 and Figure 2.5). Thus, increasing the fluid velocity by increasing the impeller speed will cause a reduction in the circulation time. Further, circulation time decreases with increasing impeller-to-tank diameter ratio (Perry *et al.*, 1998). In practise, the circulation time is taken as the time interval between two crossings in the same direction (Guérin *et al.*, 1984). In conclusion, mixing and circulation times are system and experiment-specific and the several criteria that have developed to measure these parameters are not universally applicable (Coulson *et al.*, 1999).

2.5.6.1 Factors affecting mixing times

The factors affecting mixing times are similar to those affecting mixing in general. The intensity of agitation, the method by which mixing time is obtained, the definition of mixing time (66.7% of final concentration, 80% or 99.99%), temperature, the physical properties of the fluid in use are some of the factors involved. The mixing time determined is also affected by the sensitivity and reproducibility of the method used and the vessel geometry. The intensity of agitation is defined solely by the impeller speed and the higher the intensity, the lower the mixing time (McCabe *et al.*, 2001). The impeller speed not only affects the intensity of agitation but also the viscosity of the fluid especially when a shear dependent non-Newtonian fluid is being homogenised. The temperature of the mixing vessel/content generally has an effect on the physical properties such as density and viscosity of the fluid to be mixed. The time needed for homogenising pseudoplastic fluids is much more (sometimes up to double) than that needed for Newtonian fluids (McCabe *et al.*, 2001). The reason for this difference is because of the shear rate field that exists in the mixing vessel when a pseudoplastic fluid is being mixed (Zlokarnik and Strasse, 2002). Looking at vessel geometry, Zlokarnik and Strasse (2002) concluded that as the liquid height-to-tank diameter ratio increases, the mixing time also increases.

2.5.6.2 Characterising mixing time and circulation time

When characterizing mixing time, it is important that the method and equipment used should be applicable to industrial size bioreactors, say 500l since most industrial-type stirred tank reactors are usually that big. Secondly, the method should not be limited to laboratory and research-type fermentations only so that the information gathered can be useful in industry and scale-up effects can be minimised. And finally, it should be possible to regularly obtain data throughout the fermentation (Byrant, 1977).

Byrant (1977) classified the methods of characterisation into:

- Stimulus/response techniques e.g. tracer or conductivity method
- Flow-followers and local velocity measurement
- Consumption of certain nutrients

a) Stimulus response

An application of this method is measuring the terminal mixing time. In this method, a small amount of tracer is injected into the system at time, $t = 0$ and the concentration of the tracer monitored over time. The terminal mixing time is taken as the time for the mixture to reach a certain degree of uniformity. The amount of tracer added, point of injection or pulse application and the degree of uniformity used affect the terminal mixing time determined. In the case of the bioreactors, the suitable tracer to be used should not be toxic to the microorganism, be easily detectable and measured in an aseptic manner. In any case, the laboratory broth should be "real", such that rheological properties of the broth can be taken into consideration when characterising mixing. Coulson *et al.* (1999) stated that in deciding the

point of injection, the variance of the concentration about the equilibrium value should be the major determining factor. In other words, the point of injection should be such that, the variance (standard deviation) of the equilibrium concentration should not be too high.

One of the problems associated with the tracer method is that successive addition of the same sample increases the tracer concentration thereby reducing measurement sensitivity. One way of alleviating the sensitivity problem is by adjusting the broth pH to some value between pH 7-14 and then adding phenolphthalein. The pulse on the other hand is an acid. The time taken for the pink colour to disappear is taken as the mixing time.

b) Flow-followers

This method is usually used for measuring the mean circulation time. The flow-follower is a radio follower housed in a plastic sphere. The sphere consists of two hemispheres that can be screwed together. In other cases, a tiny silicon pellet is used. It is generally accepted that in the case of vigorous mixing, the contents of the tank will have to be circulated 5 times to achieve 99% homogeneity (McCabe *et al.*, 2001). Once the mean circulation time has been obtained, the mixing time can be easily approximated from it. The problem with this method is that the level of the accuracy of the mixing time is definitely lower than that obtained by using the stimulus response method. Another limitation of this method is that it can only be used for transparent fluids where visual observation is required. In opaque fluids, a flow follower generating an electric signal through a magnetic fluid or a radar signal would be required.

2.6 LOSS OF YEAST QUALITY ON STORAGE

The loss of yeast quality can come about as a result of operating conditions which result in both cell physiological stress and mechanical stress. Variables which may influence stress experienced, include intensity of agitation of the yeast suspension, duration, temperature of storage and the physiological state of the yeast.

2.6.1 PHYSIOLOGICAL STRESS

Moderate temperatures and the lack of nutrients over a prolonged period of time results in cell starvation. Storing the yeast at very low temperatures could hinder any metabolic processes thus preventing an increase in carbon dioxide and ethanol concentrations to toxic levels and the generation of metabolic heat. Elevated carbon dioxide, ethanol concentrations and temperature can weaken the physiological condition of the cells (Smart *et al.*, 1995) thus making them more susceptible to mechanical damage. Martens *et al.* (1986) studied the influence of time and temperature of stored yeast on yeast quality and they discovered that yeast should be stored under beer at a storage temperature not exceeding 4°C. Roessler (1968) stated that one of the reasons for storing yeast at low temperatures is to prevent autolysis. It appears that the products of autolysis act as nutrients for wild yeasts and bacteria and can also affect the flavour of beer. Since the level of metabolic activity of stored yeast is temperature-

dependent, it is very important that uniform temperature is maintained. Fluctuating temperature affects the duration of the lag phase of subsequent fermentation (Roessler, 1968).

2.6.2 AGITATION

It has been reported (Lewis and Poerwantaro, 1991) that the release of haze forming material is due to mechanical abrasion of the cell wall and the amount of haze released is dependent on the pH of the medium. In the study by Lewis and Poerwantaro (1991), a brewer's strain of *Saccharomyces cerevisiae* with a dry weight concentration of 10 g/l (~1% wet weight) was agitated at 300 rpm by the use of a mechanical shaker at 20 °C for 5 min. It was also found that mature cells that had completed fermentation released more haze material than younger cells. The amount of haze material released was affected by the duration of agitation. Martens *et al.* (1986) investigated the influence of time and temperature on the quality of yeast stored and they concluded that, to maximise yeast quality, the storage vessel should not be minimally agitated. The conditions used in the study conducted by Martens *et al.* (1986) were a range of temperatures between 0 and 12 °C and storage times of between 0 and 12 days. It is not clear how this conclusion was reached because no agitation experiments were carried out.

In the study conducted by McCaig and Bendiak (1985a) in which agitated pitching yeast is compared with non-agitated yeast, they concluded that agitation caused lower viabilities and glycogen content. The pitching yeast was agitated at 300 rpm (0.72 ms^{-1}) assuming the mixing vessel is of standard geometry) for 5 days at 1°C. There was a reduction in viability because agitation led to the provision of oxygen for the cells which accelerated the metabolic process of the yeast. As a result of the accelerated metabolic process, the glycogen reserves are depleted quite quickly as they are needed for cell maintenance. Subsequently, depletion of glycogen which is the energy source leads to death and therefore lowers viabilities (McCaig and Bendiak, 1985a).

2.6.3 STORAGE DURATION

In the YCV, yeast suspension is stored for a period of between 4 - 48 hours. During this period the suspension does not have access to oxygen because a nitrogen headspace is maintained. Quain (1988) mentioned that storage of yeast would result in glycogen depletion. Glycogen is the source of energy for stationary phase yeast and any losses can easily result in cell death. In order to reduce glycogen depletion, Quain (1988) suggests that yeast handling should be conducted anaerobically and chilled. McCaig and Bendiak (1985b) conducted a study on the effect of storage duration on stored pitching yeast under beer and they observed that the viability of the yeast decreased from 96 to 89.3% over 5 days of storage and the glycogen content decreased by 35%. It was concluded that this change in viability and glycogen content is not significant enough to affect subsequent fermentations. However, they suggested that yeast should not be stored for longer than 72 hours. Sall *et al.* (1988) conducted a study on brewer's yeast (18% wet weight) stored in beer for 2 days at 1 °C and agitated continuously at an impeller tip speed of 8.51 ms^{-1} (400 rpm). The lid of their vessel was closed

with the only open space being the vent used for carbon dioxide evolution. It was concluded that glycogen and trehalose contents are not related to yeast fermentation performance. However, glycogen and trehalose declined significantly only after sampling. The conclusions drawn from the study by Sall *et al* (1988) differs from that drawn by McCraig and Bendiak (1985b). This is probably because the conditions at which they both worked was different. Martens *et al.* (1986) confirmed that storing yeast for a long period (12 days) leads to a decline in glycogen reserves (22 to 8% glycogen) and a loss of viability (97 to 88% viability). The yeast used in their study was stored under beer at 0°C.

Murray *et al.* (1984) investigated the effect of aerobic and anaerobic storage on pitching rates. They showed that glycogen level content decreased in an aerobic system as compared to the anaerobic system. In addition, the fermentation rate was faster following anaerobic storage of yeast as compared with that stored aerobically.

2.6.4 GENERATION NUMBER (RE-USE)

Yeast age is a function of the generation number. Yeast from the YCV would be reused in subsequent fermentations, hence, the reason for investigating generation number. In Nishikawa and Nomura (1974)'s work on the effect of repeated fermentation on yeast quality, it was observed that repeated fermentation leads to a gradual loss in the ability to ferment maltrotriose. Secondly, yeast flocculence is enhanced on reuse which results in poor attenuation rate. This is because of the poor quality yeast tends to flocculate in the early stage of fermentation thereby causing a decrease in the number of suspended cells. Finally, re-use of yeast can bring about an increase in the population of mutated yeast.

2.7 CONCLUSIONS

Fermentation performance is related to yeast quality. In turn, yeast quality is equally dependent on flocculation and sedimentation characteristics, yeast viability, yeast surface properties and the integrity of the cell envelope.

In order to investigate the effect of agitation on yeast quality, it is important to minimize physiological stress on the cells as this contributes to reduction in yeast quality and makes the cells more susceptible to mechanical damage. Low storage temperatures are required with good temperature control so that the yeast does not undergo any temperature abuse. The holding tank should not contain oxygen so as to minimize metabolic activity.

Loss of yeast quality may result in release of haze forming material due to damage on the cell wall. Cell disruption can release haze material as well as enzyme protease. Analysing the material can help determine the extent and location of damage to the cell. Changes in cell surface charge and hydrophobicity also indicate the loss of yeast quality. The gradual deterioration in the performance of yeast in successive fermentations is an indication of the

loss of yeast quality associated with yeast viability. This results in a reduction in biomass growth rate. Increased fluid shear stresses could be due to an increase in impeller speed or an increase in fluid viscosity. Increased fluid shear forces may cause a reduction in cell viability, lower yeast flocculence and cell disruption but not necessarily complete cell damage.

There are several factors that affect the mixing of fluids in stirred tank reactors such as: the rheology, viscosity and density of the fluid to be mixed and temperature. The viscosity of fluids can be determined by observing the relationship between shear rate and shear stress on a rheogram. Yeast suspensions have been classified as pseudoplastic at high concentrations (greater than 10% wet weight) and Newtonian at lower concentrations. Several equations such as the Vand and Einstein equations have been proposed to describe the relationship between yeast concentrations and its viscosities. Other equations have been proposed by Rudiš *et al.* (1976) and Reuss *et al.* (1979).

Mixing time is defined as the time taken for the concentration of a material to be blended in a vessel to reach a degree of uniformity following addition of a perturbation such as addition of a tracer. The factors affecting mixing times are: the intensity of agitation, the method by which mixing time is obtained, the definition of mixing time (66.7% of final concentration, 80% or 99.99%), temperature and the physical properties of the fluid in use. To promote mixing and heat transfer in a highly viscous fluid (non-Newtonian), complex impeller coupled with an impeller diameter-to-tank diameter ratio of greater than 0.5 is desirable. The use of baffles will help to promote heat transfer. On maximising heat and mass transfer there is danger of damage to yeast cells due to high shear forces. Other impellers such as radial and axial-flow impellers will not produce as much turbulence in an agitated tank containing non-Newtonian fluid.

CHAPTER 3: EXPERIMENTAL PROCEDURES

3.1 INTRODUCTION

The objective of this study is to investigate the relationship between agitation of cropped yeast during storage and yeast quality, in other words, model the yeast collection vessel (YCV) on a laboratory scale. The rheology of the yeast and the degree of mixing were determined in order to adequately quantify the extent of agitation before any experiments were conducted.

The indicators of yeast quality investigated were: viability, vitality, protease absorbance and haze formation. Measuring viability and vitality indicated the extent of cell death and the quantity or extent of cells that are metabolically inactive. Determining protease absorbance quantified the extent of minor damage to the cell membrane while the amount of haze material quantifies the extent of minor damage to the cell wall (Basson, 1996). In order to achieve the aim as defined above, cropped yeast was obtained from SAB Miller. The quality, mixing and storage conditions to which it had been subjected were ascertained before further investigations were carried out.

In this chapter the methodology used in determining the effect of agitation on yeast quality is given. Measurement of the yeast rheology and the degree of mixing in the bioreactor quantified is described in Session 3.2 and 3.3 respectively. The materials and equipment used are described in Sections 3.4 and 3.5. In Section 3.6, the YCV is compared and contrasted with the experimental rig. Section 3.7 gives a full description of the method used to expose the yeast cells to mechanical stress. Section 3.8 details the assays employed to determine the extent of quality deterioration while Section 3.9 describes the method by which cell dry weight is determined. A detailed description of the assays conducted is found in Appendix B and the composition for the chemical solutions used in these assays in Appendix A.

3.2 THE MICRO-ORGANISM

Brewer's Yeast, *Saccharomyces Cerevisiae* SAB 5 was obtained from the yeast collection vessel (YCV) of the SABMiller in Newlands (Cape Town, South Africa). The yeast used had been previously involved in the production of Castle Lager Beer, is in stationary phase and harvested at 4°C. The yeast history for each experiment is given in Appendix D.

3.3 YEAST RHEOLOGY

The apparent viscosity of the yeast suspension was measured by using the Haake VT 550 viscometer at 25°C. The viscometer uses coaxial cylinders to determine the shear stress and viscosity as the shear rate changes. There are number of sensors and the criteria used to choose a suitable sensor are: operating shear rate, temperature, and type of fluid. The sensor system used in this case is the MV-DIN rotor system (Figure 3.1). This sensor system consists of the MV cup and the DIN rotor with an annulus spacing of 1.64mm in size. MV sensors are useful for when working at medium shear rates (5-1000 s^{-1}) and DIN 53788 rotor is used for water-based suspensions. The system dimensions are recorded in Table 3.1. In order to control the temperature, the sensor is connected to a water bath. The density of the fluid was measured by the use of DMA 55 calculating density meter from Anton Paar.

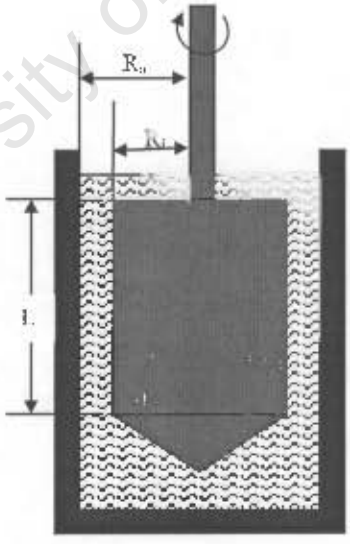


Figure 3.1: Set-up for sensor system

Table 3.1: System Properties for MV-DIN Sensor System

PROPERTY	VALUE
Inner Cylinder (Rotor):	
Radius R_i (mm)	19.36
Height L (mm)	58.08
Outer Cylinder (Cup):	
Radius R_a (mm)	21.0
Radii Ratio R_a/R_i:	
Gap Width (mm)	1.08
Sample Volume V (cm^3)	1.64
Temperature (°C)	46.0
System Factors:	
F	61.4
M	1.29

The viscometer is connected to a computer which records the shear stress at each shear rate and calculates the apparent viscosity as it changes the shear rate. The range of shear rate used was between 10 and 300 s^{-1}. To prevent settling, the data was collected within 60s. Rheology data was collected as a function of suspension concentrations ranging from 10 to 55% wet weight.

3.4 POWER REQUIREMENTS

The method by which the power measurements were made was based on the work done by Scholtz (1996). The rig used to measure the power requirements of the impeller consists of a frictionless torque table, shaft and stirrer. The impeller speed is controlled by the use of a variable speed drive. The torque table is fitted with a torque arm which coupled with a load cell is able to quantify the power used by the impeller. The load cell is calibrated by using known weights suspended on the torque arm. The power consumed could be obtained from the load cell reading via Equation 3.1.

$$P = \frac{m\,g\,L\,N\,2\pi}{60} \qquad \text{Equation 3.1}$$

where: P = power input to vessel (W)
m = load cell reading (kg)
L = distance from torque tip of torque arm to the centre of the torque table (m)
N = impeller speed (rpm)

3.5 MIXING STUDIES

Mixing studies were conducted by using 2ml of 3M KCl solution as the tracer. The tracer was injected at the bottom of the vessel (point A in Figure 3.2) as a pulse at time, t=0 and the conductivity recorded over time by the use of YSI 30-10 SCT portable conductivity meter (United Scientific, Cape Town). The calibration of the conductivity meter is described in Appendix E. The conductivity meter is adjacent to the tracer port and just below the liquid level (Figure 3.2). Bottom injection was chosen over and above top injection because the standard deviation of the equilibrium conductivity and the standard error obtained from 3 replicates were lower (Table 3.2). The average yeast concentration used in Table 3.1 is 40% wet weight. Rushton turbine at 800rpm effected the mixing.

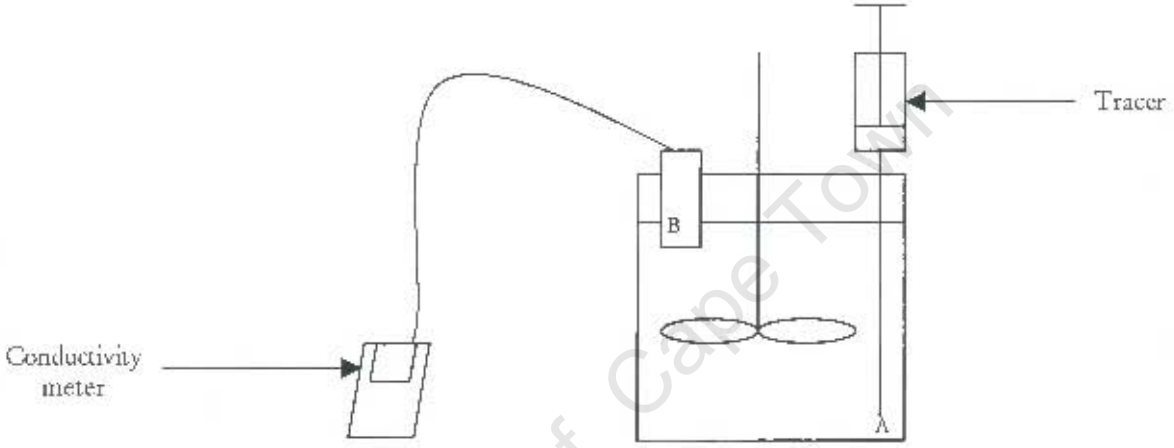


Figure 3.2: Experimental set-up for determining mixing time

Table 3.2: Difference in t_m for top and bottom injection determined at 800 rpm and yeast concentration of 40% (based on wet weight)

	Top Injection	Bottom Injection
Equilibrium conductivity (mS/cm)	0.30	0.37
Standard deviation	6.84	4.91
Standard error of 3 replicates		
$t_{m,67}$	28.4	8.4
$t_{m,95}$	28.8	8.4

Mixing time is defined as the time at which the standard deviation of the data points was between 5-10% of the final conductivity (Moo-Young *et al.*, 1972). An example is shown in Figure 3.3. The yeast suspension used to obtain the data in Figure 3.3 had a concentration of 41.9% wet weight (11.1% dry weight) and to effect mixing a Rushton turbine at an agitation rate of 400 rpm. The conductivity was monitored for between 5 and 15 minutes depending on the concentration of the yeast suspension.

Mixing time was obtained for different cell concentrations. Changing temperature, intensity of agitation and the type of agitator varied the environment of the bioreactor. The yeast

suspension considered had concentrations between 30 and 60% wet weight (8.0 – 17% dry weight). The two temperature settings used were 4 and 14°C. A Rushton turbine impeller (radial mixing) and 45° Pitched Blade impeller (axial mixing) were used to vary the type of agitation while the intensity of agitation was varied by using a range of impeller speeds between 200 and 800rpm (tip speeds between 0.72 and 2.89ms^{-1}).

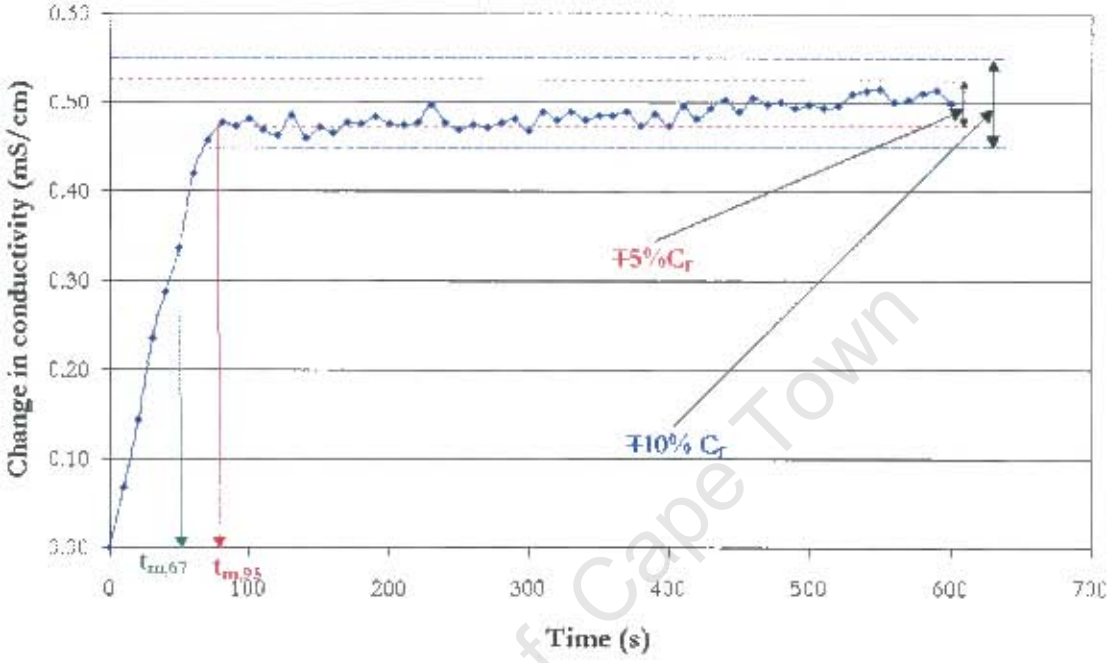


Figure 3.3: Graphical representation of data collected to determine mixing time
Impeller: Rushton turbine at 400 rpm; Yeast concentration of 41.9% wet weight

3.6 EQUIPMENT

The apparatus used to investigate the operating variables of yeast storage, as shown in Figure 3.4, consisted of a jacketed stirred tank of standard geometry. The vessel geometry is provided in Table 3.3 and the impeller dimensions and design given in Section 3.8.2.

Table 3.3: **Vessel Geometry of Yeast Agitation System**

Dimension (mm)		Dimension Ratio	
T	140.0		
H	140.0	H/T	1.00
C	53.2	C/T	0.38

The tank was a flat-bottomed 1-litre glass vessel with a sealed lid having a working volume of 0.91 litres. To ensure uniform temperature, a 25% ethylene glycol solution was recycled from a RTE-8 Endocal programmable circulating refrigerator to the vessel jacket. The temperature

of the coolant was controlled at 4°C. The apparatus was maintained under anaerobic conditions by ensuring a nitrogen headspace. The nitrogen gas was bubbled through distilled water to humidify it. The vessel contents were agitated with the use of either a Rushton or pitched-blade impeller fitted to a stainless steel shaft and a Heidolph Stirrer (Labotec, Cape Town, Republic of South Africa).

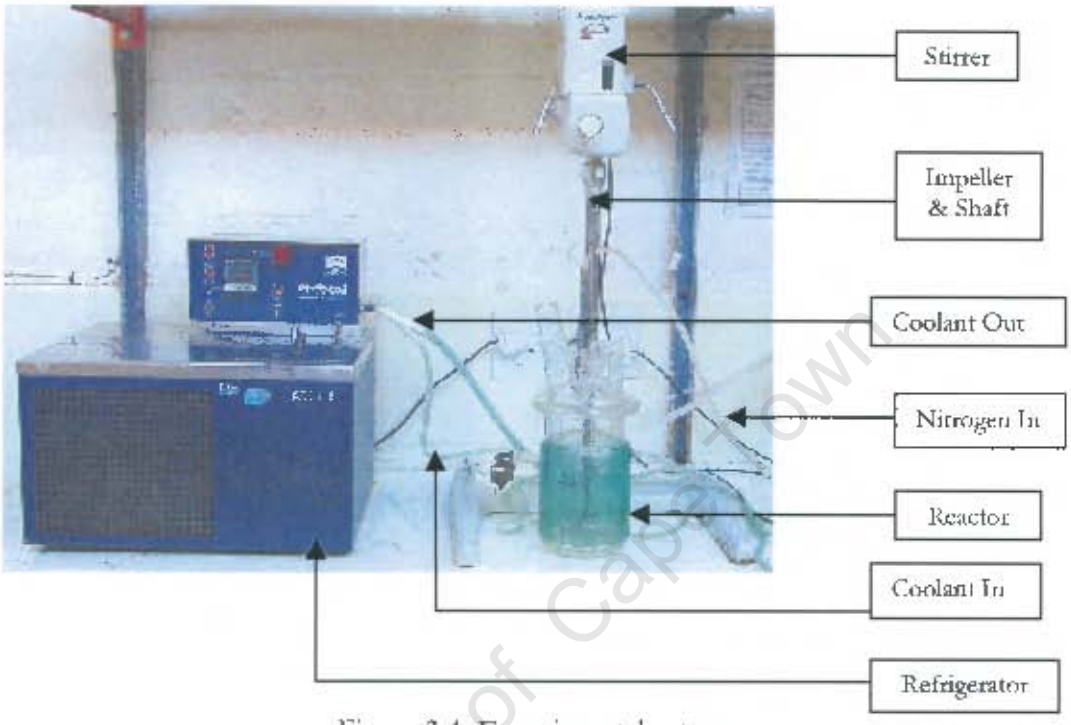


Figure 3.4: Experimental set up

3.7 COMPARING YCV AND EXPERIMENTAL RIG

The YCV is a cylindroconical vessel that is used to store cropped yeast for between 4 and 48 hours after the brewery fermentation is complete and before re-use in another fermentation. Before the suspension is stored in the YCV, it is cooled from 14 to 4°C in a plate and frame heat exchanger. The vessel and impeller dimensions for the yeast collection vessels used at SABMiller Newlands Brewery and the laboratory experimental rig are reported in Table 3.4. Agitation rates for the experimental system were chosen to provide common impeller tip speeds with those used at Newlands Brewery. The impeller type used in the YCV is the 2-bladed Scandi brew impeller. Reynolds Number (N_{Re}), shear rate and apparent viscosities are compared across the different vessels and yeast concentrations in Table 3.5. The shear rates shown in Table 3.5 were calculated from Equation 2.2. At low concentrations (< 15.0% wet weight), the yeast suspension exhibits the rheology of a Newtonian fluid and at high concentrations, the rheology is non-Newtonian, hence the reason for calculating N_{Re} for different yeast concentrations.

Table 3.4: Vessel and Impeller Dimensions for the YCV and Rig

Dimensions	YCV	Experimental Rig
T (m)	1.8	0.14
H (m)	4.50	0.14
C (m)	1.80	0.05
H/T	2.50	1.00
C/T	0.40	0.38
N (rpm)	60 - 100	200 - 800
Tip speed (m/s)	1.79 - 2.98	0.72 - 2.89
D (m)	0.570	0.069
W (m)	0.115	0.014
W/D	0.20	0.21

Table 3.5: Rheology parameters for the YCV and Rig

Yeast concentration (%)	Rheology Parameters	YCV	Experimental Rig
20.0	Shear rates (s^{-1})	9.40 - 14.1	47.0 - 188
	Apparent viscosity (cP)	10.2 - 10.0	9.58 - 9.09
	Reynolds Number	32400 - 55000	1650 - 6970
27.3	Shear rates (s^{-1})	9.24 - 13.9	46.2 - 185
	Apparent viscosity (cP)	50.4 - 42.9	26.5 - 15.2
	Reynolds Number	7580 - 15500	592 - 4120
44.0	Shear rates (s^{-1})	9.18 - 13.8	45.9 - 184
	Apparent viscosity (cP)	260 - 214	120 - 61.9
	Reynolds Number	1520 - 3230	132 - 1020
66.3	Shear rates (s^{-1})	8.14 - 12.2	40.7 - 163
	Apparent viscosity (cP)	2785 - 1904	616 - 168
	Reynolds Number	171 - 459	25.7 - 377

3.8 EFFECT OF OPERATING VARIABLES ON YEAST QUALITY

The operating variables investigated are impeller speed and type, vessel temperature, duration of agitation and suspension concentrations.

3.8.1 METHODOLOGY

Yeast was collected from the YCV of SABMiller Brewery at Newlands and transported in a sealed bottle to University of Cape Town within 15 minutes. By the time the yeast suspension reached its destination, its temperature had increased from 4°C to 10°C. The suspension is then stored in a refrigerator at 6°C. It is important that the yeast is used within a 24-hour period in order not to introduce stress due to storage. An aliquot of 0.91 litres of yeast suspension placed in the reactor. The pH, viscosity, density and biomass concentration of the slurry were measured and the physical condition of the yeast was not altered. To ensure that

the physical conditions of the yeast is not altered, the temperature was kept constant by the use of a refrigerator and oxygen was kept at bay by maintaining a nitrogen headspace. The temperature was kept constant at 4°C throughout each run of an 8 and 24-hour duration. Samples were taken and analysed every 2nd hour to monitor the yeast quality.

3.8.2 EFFECT OF IMPELLER TYPE AND SPEED ON YEAST QUALITY

To investigate the effect of impeller type on yeast quality, experiments were carried out with two types of impellers. These were:

- 6-bladed Rushton impeller and
- 6-bladed pitched blade impellers (45°).

The dimensions of these impellers are shown in Table 3.6.

These two impellers were chosen because they vary in the type of mixing they create in the suspension (See Figures 2.4 and 2.5). The Rushton impeller gives radial mixing projecting the fluid towards the vessel walls while the pitched blade impeller gives an axial flow pattern. The Rushton impeller is expected to induce more shear than the pitched blade impeller. Consequently, the Rushton impeller draws more power than the pitched blade impeller.

Table 3.6: Impeller Geometry for Experimental Rig

Dimension (mm)			Dimension ratio		
Impeller Type	Rushton	Pitched Blade		Rushton	Pitched Blade
D	68.6	70.0	D/T	0.49	0.50
L	17.8	25.9	L/D	0.26	0.37
W	14.4	11.2	W/D	0.21	0.16
H_u	46.0	20.3	H_u/D	0.67	0.29
Angle	——	45°			

The impeller speed was varied between 200 and 800 rpm (tip speed of 0.72 – 2.90 s^{-1}). The speed was varied for both impeller types and its value measured by the use of a hand-held Lutron DT-2236 tachometer (Merck, Cape Town). The Reynolds Number, shear rate, apparent viscosity, impeller tip speed and P/V for the two impeller types at 400 and 600 rpm and a 55% wet weight yeast suspension are tabulated in Table 3.7.

Table 3.7: Distinction between Rushton turbine and pitched blade impeller

Parameters	Rushton Turbine		Pitched Blade Impeller	
Impeller Speed (rpm)	400	600	400	600
Impeller tip speed (m/s)	1.44	2.16	1.47	2.20
Shear rate (s^{-1})	90.3	138.3	89.1	135.8
Apparent viscosities (cP)	267	202	269	204
Reynolds Number	119	239	121	241
P/V (kW/m^3)	1790	5466	770	2336
Cell concentration (% wet weight)	55.0	54.9	55.0	54.9

3.8.3 EFFECT OF THE DURATION OF AGITATION

In order to observe the effect of long-term agitation on the quality of yeast, a number of 24-hour experiments were carried out in which case samples were taken every 2 hours for the first 8 hours and the last eight hours. This was conducted with the Rushton turbine and at two speed settings: 400 rpm and 800 rpm.

3.8.4 DILUTION

Yeast was diluted in phosphate-buffered saline (PBS) solution to adjust its biomass concentration and the same experiments as that carried out with the undiluted yeast were performed. The biomass concentrations investigated were across a consistency range between 30 and 78% based on wet weight (Dry weight range is between 11 and 23%). This way the combined effect of dilution and suspension concentration during storage on quality was studied.

3.8.5 EFFECT OF TEMPERATURE

Two temperature settings were used in this set of experiments: 4 and 14°C. The temperature at 4°C was used because this is the storage temperature at which the YCV is maintained while the rationale for using 14°C is because this is the temperature from which the yeast is cooled down from before it enters the YCV.

3.9 YEAST QUALITY

The yeast was assessed using the following yeast quality indicators:

- Cell viability
- Haze material
- Protease
- Vitality

3.9.1 CELL VIABILITY

There are a number of ways by which yeast cell viability can be determined. These methods have been classified into three: cell damage, loss of metabolic activity and loss of replicative ability (Jones, 1987 in McCaig, 1990).

Examples of methods that use direct measurement of loss of replication as their principle are the plate count and slide culture techniques. McCaig (1990) reports that these methods are slow (requires 18-48 hours), prone to error (plate counts) and pose a problem when diluting the cell clumps in liquid media.

Dyes and stains use the principle of cell membrane damage to determine cell viability. These stains are dependent on the state of the plasma membrane (McCaig, 1990). Examples of such dyes and stains are Mg-ANS, eosin-Y, methylene blue and methylene violet to mention a few. Mg-ANS stains require a fluorescent microscope while methylene blue dyes need a brightfield microscope. Non-viable cells are stained on addition of Mg-ANS while viable cells remain unstained (King *et al.*, 1991). The principle behind the fluorescent stain is that the nonviable cells allow the stain into their cytoplasm, the stain then binds to the cytoplasmic protein and the resultant complex formed as a result of this fluoresces, giving off a solid bright green colour when illuminated by UV light (McCaig, 1990). However, in viable cells, the stain binds to the exterior protein of the cell wall and gives off a halo of weak green fluorescence on illumination (McCaig, 1990).

The methylene blue staining technique relies on the permeability of the cell membrane to ensure staining. Dead cells have a porous membrane that allows the methylene blue solution to be absorbed whereas living cells would not be stained by the solution. Although methylene blue staining method is commonly used in industry (Lentini, 1993), it has its limitations in that it overestimates the viability when the viability is less than 90% (King *et al.*, 1981). McCaig (1990) relates this to the subjective nature of this technique in that staining can be very light which can be recorded as either positive or negative. Methylene blue stain can undergo oxidative demethylation which is the reason why it gives differences in staining intensities. As a result Smart *et al.* (1999) recommended the use of citrate methylene violet dye. Citrate methylene violet dye has an advantage over the traditional methylene blue stain because it has the ability to make a better distinction between living and dead stains as well as having a neutral pH. The methylene blue staining method has the advantage over the Mg-ANS staining method in that, it is inexpensive and simple. Although, it must be noted that in the lower viability range (<90%), the Mg-ANS staining method gives more accurate results when compared with the methylene blue staining method.

In this study, the modified methylene blue staining technique of Lee *et al.* (1981) was employed, as the viability is not expected to fall below 90%. Lee *et al.* (1981) suggested the use of Ringer solution to prevent cell growth.

To determine cell viability, the number of stained (non-viable) and unstained (viable cells) was counted by the use of a Neubauer haemocytometer, a light microscope with a 40x magnification and a Han Lien DBC-6 counter. Buds were only included in the count if they are bigger than 50% of the size of the mother cell based on area. Cell viability is then calculated in the following manner (Equation 3.2):

$$\%\ cell\ viability = \frac{viable}{viable + non - viable} \times 100 \qquad \text{Equation 3.2}$$

The coefficient of variance for this method was calculated to be 1.1%. The reproducibility of the method and its practice are given in Section 4.3.1 and Appendix B.1.1 respectively.

3.9.2 PROTEASE

There are a number of methods used to determine extracellular protease activity, including those proposed by Mochaba *et al.* (1993) and Ormrod *et al.* (1991). In the method of Mochaba *et al.* (1993), the substrate used is resorufin-labelled casein whereas the method by Ormrod *et al.* (1991) uses bovine haemoglobin as the substrate. The haemoglobin method is not sensitive at low levels of protease activity and the protease activity measured by this method is not reproducible (Ormrod *et al.*, 1991). However, the casein method is highly sensitive, reproducible and a direct link between the protease activity detected and the cell viability is reported (Mochaba *et al.*, 1993). Furthermore, the method is simple.

Robinson (2001) modified the method proposed by Mochaba *et al.* (1993) by proposing an incubation time of 1hr. To ensure the linearity between protease present and absorbance, it is necessary to operate in the linear response range as samples with different levels of protease are being compared (Robinson, 2001). The method used in this study is that proposed by Robinson (2001). A description of the method is in Appendix B.4.1 and its reproducibility is reported in Section 4.3.2. The coefficient of variance for this method is 4.0%.

3.9.3 HAZE MATERIAL

An increase in the amount of haze in beer can affect its taste, its appearance through turbidity and therefore affect its quality. Siebert *et al.* (1981) observed that the presence of haze in beer poses a problem in beer clarification. Lewis and Poerwantaro (1991) noticed an increase in haze on agitation. Haze forming materials can include carbohydrates, proteins, polyphenols, metal ions, oxalate or even microorganisms. Thus, an increase in haze in a suspension can be caused by a number of factors such as: a change in pH, starvation and temperature stress, as well as mechanical stress.

Siebert *et al.* (1981) tried to characterise haze by using the following methods:

- Nephelometry
- Chemical analysis
- Enzyme treatment
- Optical microscopy with staining
- Scanning electron microscopy and
- Electron particle counting

Of all these methods, nephelometry and electronic particle counting gave the better results i.e. the amount measured correlated well with the amount of haze collected by centrifugation. The problem with electronic particle counting is that if the sample is not handled properly (kept cold), the amount of haze is susceptible to change. However, if sample is well handled, electronic particle counting gave more accurate results than nephelometry.

For this study, haze determination was based on particle counting but used the Malvern Mastersizer (long bed version 2) to quantify the amount of haze in the supernatant of a sample by laser light scattering in place of the electronic particle counter. The Malvern mastersizer is able to detect particles having sizes within the range of 0.05 to 900 μm using the principle of laser light scattering to detect particles. The larger the particles, the more it scatters or deflects the light. The amount of deflection or scatter is used to calculate the particle size. The procedure for haze determination by laser light diffraction was developed by Robinson (2001). Haze material is considered to be composed of any particles smaller than 2 μm (Robinson, 2001). A full description of the haze characterisation method is given in Appendix B.3.1. The reproducibility of the method is clearly described and explained in Section 4.3.4. The coefficient of variance for this method is 2.1%.

3.9.3.1 Quantification of Haze

Haze has been classified as particles in the size range of 0.08 to 2 μm (Fischer *et al.*, 2001). However, particles in the size range of 2 to 3 μm are protein-polyphenol compounds (Fischer *et al.*, 2001). In order to quantify haze, a size distribution plot is obtained from the Mastersizer. The size distribution plot obtained from the Mastersizer is on a volume basis and Robinson (2001) suggests that the data is converted to a particle number or area basis (assuming the particles are spherical) in order to avoid the smaller haze particles from being overshadowed by bigger particles such as the yeast cells and agglomerates. For this study, the data obtained from the Mastersizer was left as volume basis because the data obtained were to be compared and normalised with the control data. The area under the size distribution curve was calculated by using Sigma Plot toolkit which uses the integral principle to evaluate the area under the graph. The ratio of the area under the size distribution curve in the haze size range i.e. particle diameter less than 2μm (A_2) as shown in Figure 3.5a and the area under the size distribution curve in the entire range (A_T) as shown in Figure 3.5b was then calculated by using Equation 3.3.

$$Amount\ of\ haze = A_2 / A_T \qquad \text{Equation 3.3}$$

In some cases, the data was normalised by comparing the amount of haze obtained from the agitated yeast with that obtained from the control yeast (Equation 3.4)

$$Normalised\ haze = \frac{Amount\ of\ haze\ in\ treated\ yeast\ (H_t)}{Amount\ of\ haze\ in\ control\ (H_c)} \qquad \text{Equation 3.4}$$

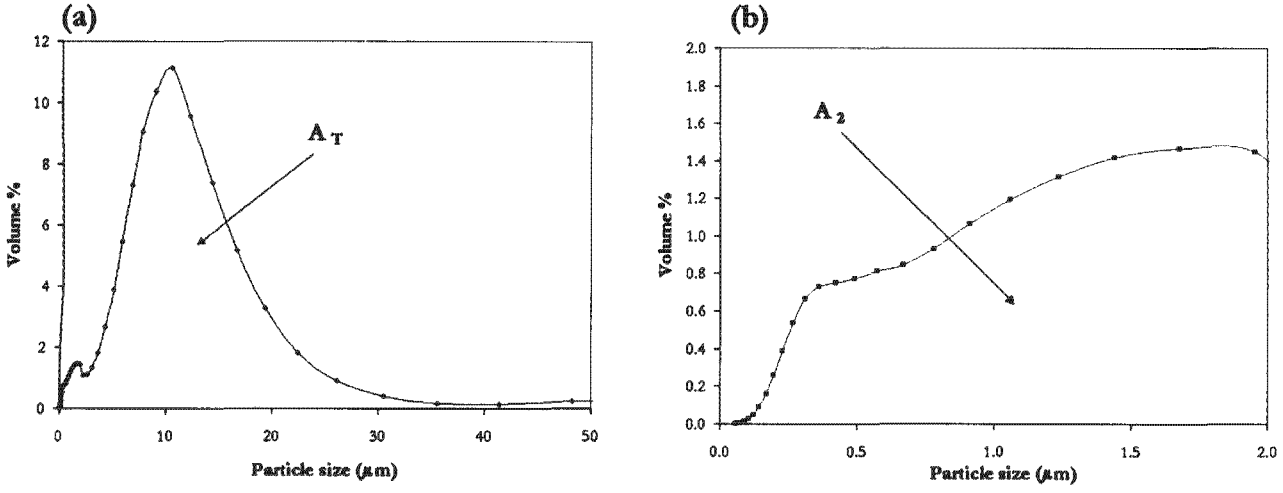


Figure 3.5: Haze analysis. (a)- Size range (0 – 50 µm) (b)- Size range (0 – 2µm)

3.9.4 VITALITY:

Yeast vitality can be characterised in terms of metabolic activity, cellular components, its fermentation capacity, acidification power and its oxygen uptake ability (Lentini, 1993). In this study, yeast vitality was determined by analysing the fermentation capacity of the stressed yeast. An amount of yeast was grown anaerobically in a shake flask for 24 hours at an agitation rate of 150rpm at 30°C and changes in biomass concentration, viability; CO_2 evolved, and substrate concentration were measured every 2nd hour for the first 8 hours and thereafter at 24 hours. Sampling was done aseptically in the laminar flow cabinet. Although this method is time consuming, it gives a direct indication of the yeast vitality (Lentini, 1993), unlike the indirect measures given by abundance of cellular compounds or by acidification power. A more detailed description of the vitality method is given in Appendix B.5.1.

3.9.4.1 Glucose concentration

The cells were grown on Maltose Yeast Peptone and Glucose (MYPG) media (Appendix A.3.1) and hence, the major carbon source used is glucose. Glucose concentration was measured by determining the reducing sugar concentration using 3,5-dinitrosalicylic acid (DNS). The composition of the DNS solution is given in Appendix A.3.2. In this method known as the dinitrosalicyclic colorimetric method of glucose determination (Wang, 2003), the presence of free carbonyl groups (C=O) is tested. The carbonyl groups are oxidised as the DNS is reduced to 3-amino,5-nitrosalicyclic acid. This occurs in alkaline conditions. The method is described in Appendix B6. The coefficient of variance for this method is 1.7%.

3.9.4.2 Cell concentration

The cells were counted as described in Section 3.9.1 and Appendix B.2.1. Biomass concentration is the product of the number of cells counted, the dilution factor and the volume of suspension present with the area of the haemocytometer used ($25mm^2$). The coefficient of variance for this method is 3.7%.

3.9.4.3 Carbon dioxide evolved

The shake flask was fitted with a rubber bung through which a glass rod filled with silica gel was inserted. The silica gel absorbed any moisture from evaporation of the medium, ensuring that any weight loss can be attributed to carbon dioxide evolution only as confirmed by the control flask. The flask was weighed every 2nd hour for the first 8 hours and at 24 hours. The coefficient of variance for the method is 18.6%.

3.9.4.4 Analysis of Vitality data

Vitality can be measured by the yield of biomass and carbon dioxide with respect to substrate consumed, growth rates, substrate utilisation rates and carbon dioxide formation rates of a growing cell. Some typical vitality data are shown in Figures 3.6, 3.7, 3.8 and 3.9. The vitality of yeast cells that had been exposed to stress on storage for 7 days (treated yeast) and that of cells that had not been subjected to storage at all (untreated yeast) is compared. Cells were stored in a closed bottle at 6°C in a refrigerator.

Cell growth is seen to start after 2 hours (Figure 3.6a). Using the period from 2 to 8 hours, the growth rate can be calculated based on Malthus kinetics (exponential growth). The growth rate for the treated yeast cells as calculated from Malthus equation for batch kinetics (Equation 3.5), is half that of untreated yeast cells (Figure 3.7).

$$\mu_x = \frac{1}{X}\frac{dX}{dt} \qquad \text{Equation 3.5}$$

There is only a slight difference in the substrate utilisation of the stored yeast and fresh yeast suspension. However, the amount of carbon dioxide formed by the fermentation of the untreated yeast cells is 6.7% more than that formed by the treated yeast cells.

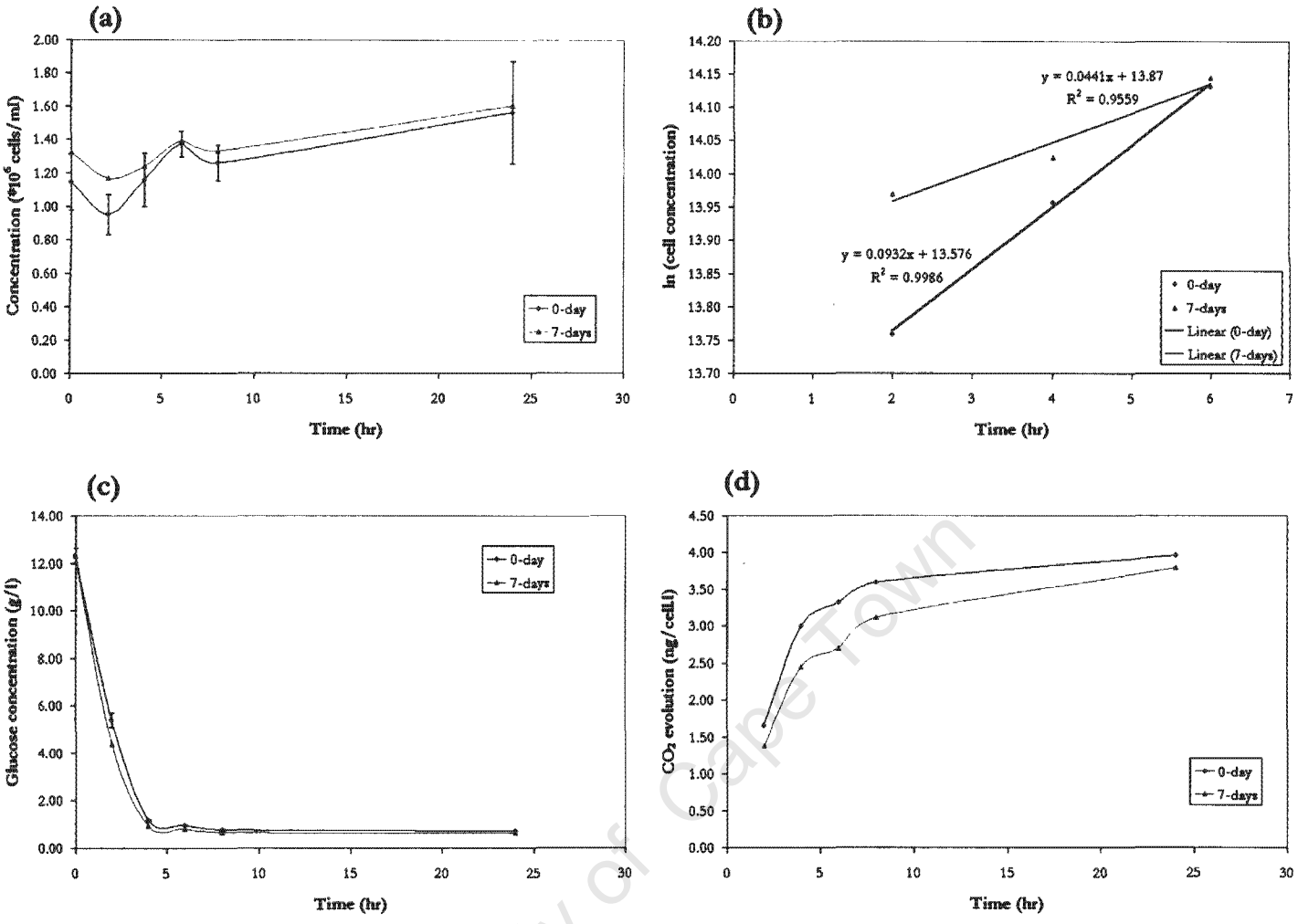


Figure 3.6: Vitality profiles for cells that had been stored for 0 and 7 days.
(a)-Growth profiles; (b)-Growth rates; (c)-Glucose utilisation; (d)-Carbon dioxide formation

The yield of biomass on glucose ($Y_{X/S}$) and the yield of carbon dioxide on substrate ($Y_{CO2/S}$) are calculated from Equations 3.6 and 3.7 respectively while, substrate utilisation rate (q_S) is calculated from Equation 3.8. Substrate utilisation rates, yield of biomass and carbon dioxide have been calculated for exponential phase (exponential) and the entire growth period (average).

$$Y_{X/S} = \frac{Final\ cell\ concentration\ (*10^6\ cells.ml^{-1})}{Glucose\ consumed\ (g.l^{-1})} \quad \text{---------- Equation 3.6}$$

$$Y_{CO_2/S} = \frac{Amount\ of\ CO_2\ produced\ (ng/cell.ml^{-1})}{Glucose\ consumed\ (g.l^{-1})} \quad \text{--------- Equation 3.7}$$

$$q_S = \frac{1}{X}\frac{d[S]}{dt} \quad \text{--------- Equation 3.8}$$

Table 3.8: Growth Parameters for treated and untreated yeasts

Growth Parameter	Untreated Yeast	Treated Yeast
$Y_{X/S}$ (*10^9 cells/g):		
Average	0.04±0.02	0.02±0.01
Exponential	0.09±0.03	0.04±0.02
$Y_{CO2/S}$ (ng CO_2/g.cell):		
Average	0.34±0.05	0.33±0.04
Exponential	0.78±0.10	0.84±0.20
qs (g/cell.h):		
Average	-0.42±0.05	-0.37±0.03
Exponential	-0.78±0.07	-0.54±0.15

These growth parameters have been calculated for treated and untreated yeasts and are presented in Table 3.8. The difference between the yields on biomass for untreated and treated yeast is quite significant (52.7% decrease on treatment) and there is a 25% difference in the exponential substrate utilisation rate. However, there is only a 12% difference in the average substrate utilisation rate and a 7.7% increase in the yield on carbon dioxide.

3.10 DRY-WEIGHT ANALYSIS

Between 1.0 and 1.5 ml of yeast sample is placed in a dried and pre-weighed (w_1) Eppendorf microfuge tube and weighed (w_2). The sample is then centrifuged for 5 min at 10 000rpm, washed with distilled water and centrifuged again at the same conditions. This is repeated 2 to 3 times till the supernatant becomes clear. The wet weight of the yeast was determined by weighing the tube at this stage (w_3). The tube was then dried at 80°C in the Econo 38 801 Labotec Oven for between 12 and 48 hours to constant mass (w_4) depending on sample concentration. This was done in triplicate. Wet weight and dry weights were calculated from Equations 3.9 and 3.10 respectively.

$$Wet\ weight = \frac{(w_3 - w_1)}{(w_2 - w_1)} * 100 \quad \text{——————} \quad \text{Equation 3.9}$$

$$Dry\ weight = \frac{(w_4 - w_1)}{(w_2 - w_1)} * 100 \quad \text{——————} \quad \text{Equation 3.10}$$

The coefficient of variance for 5 single samples is 3.7%. A full statistical analysis on this method is shown in Section 4.3.3.2.

3.11 CONCLUSIONS

The aim of this study is to simulate the YCV on a laboratory scale and check the effect of agitation on yeast quality. The yeast used in this case is that of the specie *Saccharomyces cerevisiae* and this was obtained from the Newlands Brewery of SABMiller. Agitation was provided by the use of two impellers: Rushton turbine and 45° pitched-blade impellers and the vessel used had standard geometry. The physical properties of the yeast such as its density and viscosity respectively were determined by the use of an AP densitometer and a Haake viscometer. The type of rheology that the yeast suspension exhibits was investigated in order to establish the type of mixing that would be experienced in the mixing vessel.

A correlation between certain variables and the indicators of yeast quality were to be established. Such variables are: storage temperature, cell concentration, impeller speed and type and duration of agitation. Further, the indicators of yeast quality are: viability, yeast performance (vitality), the amount of haze and protease. These indicators of yeast quality were measured by the use of various assays. In the case of viability, a number of possible assays were highlighted and the methylene blue staining method was chosen over above the other methods mainly because of its ease of application and because the viabilities being measured are in the range in which the method is suitable. Yeast performance was quantified by subjecting the yeast to anaerobic growth on MYPG media at 30°C and measuring the growth rate, yields on biomass and carbon dioxide and substrate utilisation rates. Finally, the amounts of protease and hazes were measured by the use of the method by Robinson (2001) and the Mastersizer respectively. The Mastersizer counts the number of particles in a particular size range by the use of laser diffraction.

CHAPTER 4: REPRODUCIBILITY OF RESULTS

4.1 INTRODUCTION

This chapter deals with the reproducibility of the many experimental assays employed and the experiments conducted. Statistical analysis tools such as the ANOVA, t-test and f-test were used to interpret the significance of the results. These tools were obtained from the Excel analysis toolkit. The definitions of these statistical tools are given in Section 4.2. Data are said to be reproducible if the confidence level for difference is less than 90% or the probability is greater than 0.01, otherwise the data being compared are statistically different.

4.2 DEFINITION OF STATISTICAL TOOLS

The statistical tools used in this study are the t-test, f-test and the ANOVA (analysis of variance). Other statistical parameters used are the average, standard deviation, standard error and confidence limits.

4.2.1 AVERAGE, STANDARD DEVIATION AND STANDARD ERROR

The average for a population gives an indication of where most of the data lie. However, the standard deviation of a population shows how accurate the set of data is and the standard deviation is replaceable by the standard error when comparing large datasets (Napier-Munn, 1994). For a set of variables, x_1, x_2, x_3, ... x_n, the average ($\bar{x}$), standard deviation (s) and standard error ($s_{\bar{x}}$) for these data are given in Equation 4.1, 4.2 and 4.3, respectively.

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \qquad \text{Equation 4.1}$$

$$s = \sqrt{\sum_{i=1}^{n} \frac{(x_i - \bar{x})}{n-1}} \qquad \text{Equation 4.2}$$

$$s_{\bar{x}} = \frac{s}{\sqrt{n}} \qquad \text{Equation 4.3}$$

4.2.2 CONFIDENCE LIMITS

Confidence limits refer to the probability that the set of data being compared is different.

4.2.3 t-TEST

The t-test is a statistical tool that is used to test the significance of the difference between two means of two populations. Napier-Munn (1994) states that the t-test is only valid if the variances of the populations being compared are not significantly different, the distribution of the data is normal and the characteristics of the sample populations are constant. Furthermore, each data point in the population needs to be an independent and an unbiased sample. For two population sets with sample sizes n_1 and n_2, standard deviations; s_1 and s_2 and means; $\bar{x}_1$ and $\bar{x}_2$, the t-test can be applied by calculating t and s.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{1/n_1 + 1/n_2}} \qquad \text{Equation 4.4}$$

where: $$s = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}$$ ——— Equation 4.5

The confidence limits on the difference is given by:

$$\pm t\, s \left({}^{1}\!/_{n_1} + {}^{1}\!/_{n_2} \right)^{0.5}$$ ——— Equation 4.6

In a case in which time-dependent variations are being tested between two methods, the t-test is applied by using:

$$t = \frac{\bar{d}}{s_d / \sqrt{n}}$$ ——— Equation 4.7

where:

s_d	=	standard deviation of time-dependent data
n	=	population size
$\bar{d}$	=	mean difference = $\frac{\sum d_i}{n}$
d_i	=	difference = $x_1 - x_2$

$$s_d = \sqrt{\frac{\sum_{i=1}^{n} (d_i - \bar{d})^2}{n-1}}$$ ——— Equation 4.8

The confidence limit in this case is given by:

$$\pm \frac{t\, s_d}{\sqrt{n}}$$ ——— Equation 4.9

where t is obtained from the t-tables and is a function of the level of confidence and the degree of freedom (n-1).

4.2.4 F-TEST

The f-test is useful for comparing two variances (Napier-Munn, 1994). As mentioned earlier, the t-test can only be used in cases where there is no significant difference in the variances implying that the f-test is a prerequisite for the t-test. Equation 4.10 is used for the f-test.

$$F = s_1^2 / s_2^2 \text{ ---------- Equation 4.10}$$

with s_1 being the larger standard deviation i.e. $F \geq 1$.

The value of F calculated is compared with F_t obtained from the F-table. The degrees of freedom have been calculated as n_1-1 and n_2-1. For the difference not to be significant, $F_t > F$ and vice versa.

4.2.5 ANALYSIS OF VARIANCE (ANOVA)

The ANOVA is a statistical tool that is used to assess the significance of several variances. This is done by using the f-test to analyse the variances of the means (Napier-Munn, 1994). Aside from its usefulness in determining the significance of several means, the ANOVA can also be used to analyse the data obtained from factorial experimental designs and randomised blocks.

In a case where the viabilities of two fermentation experiments are being compared, there will be three possible sources of variations: fermentation, time and error. Thus, these variations can be evaluated by the sum of squares (SS) which for fermentation is given by Equation 4.11.

$$SS_f = \sum_{i=1}^{k} n_i \left(\bar{x}_i - \bar{x}\right)^2 \text{ ---------- Equation 4.11}$$

where:

k	=	number of fermentations
$\bar{x}$	=	overall data mean
$\bar{x}_i$	=	mean of the i^{th} fermentation
n_i	=	number of replicates for the i^{th} fermentation

The residual sum of squares after all the variations have been accounted for is the sum of squares due to error and is given by Equation 4.12.

$$SS_o = \sum_{j=1}^{n_1} \left(x_{1j} - \bar{x}_1\right)^2 + \ldots\ldots + \sum_{j=1}^{n_k} \left(x_{kj} - \bar{x}_k\right)^2 \text{ ---------- Equation 4.12}$$

where: x_{ij} is the j^{th} observation for the i^{th} fermentation. The total sum of squares is the sum of the variations (sums of squares) due to fermentation, time and error.

An example of a two-way ANOVA in which two or more factors are investigated, calculated by the use of Excel Analysis tool pack is given in Figure 4.1.

Anova: Two-Factor Without Replication for yeast collected on different days

Sampling time (hr)	Count	Sum	Average	Variance
0	2	195	97.6	0.045
1	2	194	96.9	0.011
3	2	196	98.0	0.216
4	2	196	98.1	0.007
6	2	195	97.6	0.027
Day 1	5	488	97.6	0.207
Day 2	5	488	97.7	0.307

ANOVA

Source of Variation	SS	df	MS	F	P-value	F_{crit}	Confidence Level (%)
Rows (Sampling time)	1.77	4	0.441	6.08	0.054	6.39	94.6
Columns (Experiments)	0.015	1	0.015	0.207	0.673	7.71	32.7
Error	0.291	4	0.073				
Total	2.07	9					

Figure 4.1: An example of Two-way ANOVA without replication

where:

SS	=	Sums of squares
df	=	degrees of freedom
MS	=	Mean squares
F	=	Calculated f-value
F_{crit}	=	F-value obtained from F-table
P-value	=	Probability that there is no significant difference

In Figure 4.1, it is seen that F is much less than F_{crit} in looking at the variations between experiments, hence the reason for the low probability value (32.7% probability). Further, there is a 94.6% probability that there is a significant difference between samples collected at different times. In conclusion, there is no variation between experiments but the time at which the sample is collected is very important.

4.3 EXPERIMENTAL VARIATIONS

The yeast suspensions used in the experiments were collected from the SABMiller Brewery in Newlands, South Africa on different days of the week. As it was difficult to carry out experiments on the same day and on the same yeast sample due to the constraints of the number of experiments to be conducted, samples were collected on different days and experiments carried out on yeasts with different histories. The histories of yeast samples have been classified using the day of sample collection and their generation numbers. The variation in the samples were statistically analysed using ANOVA and the samples were said to be significantly different at the 90% confidence level if their probability is greater than 0.1.

4.3.1 VARIATION BETWEEN YEAST COLLECTED ON DIFFERENT DAYS

Two sets of experiments, conducted using a Rushton impeller at a speed of 400rpm with the refrigerator temperature set at 14°C, were compared. The viabilities of these runs were used to determine if there are variations between experiments. The sets of data compared had a wet weight concentration of 45.0%. The confidence level that a difference existed between these experiments was calculated to be 32.7%; hence it can be concluded at the 90% confidence level sought that there is no significant difference between experiments (Appendix C1).

4.3.2 VARIATION BETWEEN YEAST GENERATIONS

In these experiments, yeast suspension had been collected across a range of generation number (i.e. cycles of re-use within the brewery). Two analyses are presented here to investigate the influence of yeast generation number. In the first case, yeast cream having a wet weight concentration of 62% was subjected to mechanical stress at an impeller speed of 400rpm with a Rushton turbine in two experiments. The only difference between these was that the yeasts were of different generations: 1st generation and 4th generation. The ANOVA data are presented in Figure 4.2. In another scenario, the viabilities of 4th and 7th generation yeast cells were compared (Figure 4.2). These yeast suspensions had a concentration of 55.0% wet weight and they were agitated at 200 rpm using Rushton turbine at 4°C. In both cases, there was no significant difference between the viabilities measured across different generations. The confidence levels were calculated to be 32.3% and 66.7% respectively.

Anova: Two-Factor Without Replication for Yeast Generations

Scenario 1

Sampling time (hr)	Count	Sum	Average	Variance
0	2	190	94.9	1.07
1	2	185	92.5	8.64
4	2	194	97.2	0.108
6	2	191	95.3	1.92
8	2	183	91.6	4.72
Generation 1	5	470	94.0	6.07
Generation 4	5	473	94.6	7.75

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit	Confidence Level (%)
Rows (Sampling times)	39.6	4	9.91	2.53	0.195	6.39	80.5
Columns (Generation)	0.785	1	0.785	0.201	0.677	7.71	32.3
Error	15.7	4	3.92				
Total	56.1	9					

Scenario 2

Sampling time (hr)	Count	Sum	Average	Variance
0	2	195	97.6	0.015
1	2	196	98.2	0.162
3	2	191	95.3	8.30
4	2	195	97.6	0.199
6	2	194	97.1	0.376
Generation 4	5	488	97.6	0.443
Generation 7	5	484	96.7	3.66

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit	Confidence Level (%)
Rows (Sampling times)	9.47	4	2.37	1.36	0.386	6.39	61.4
Columns (Generation)	2.10	1	2.10	1.21	0.333	7.71	66.7
Error	6.95	4	1.74				
Total	18.5	9					

Figure 4.2: Two-way ANOVA for testing variations between yeast generations

4.4 REPRODUCIBILITY OF METHODS

Through analysing between 4 and 6 equivalent samples using the analytical method in question, the reproducibility of the methods was tested. The average, standard deviation from the mean and the coefficient of variance were calculated and in some cases, the two-way ANOVA was used.

4.4.1 VIABILITY

Table 4.1 shows the average, standard deviation and coefficient of variance of 5 readings of same sample for the methylene blue staining method. Robinson (2001) found the coefficient of variance for the methylene blue staining method to be 0.95%. The coefficient of variance for this method is 1.0%. The confidence limit of the viabilities at 95% confidence level was calculated to be 1.1%. Hence, the difference between two viabilities will be considered to be statistically significant if it is greater than 1.1%.

Table 4.1: Viability Statistics

Sample No.	Viability (%)
1	94.0
2	93.2
3	92.9
4	93.4
5	95.2
Average	93.7
Confidence limit at 95% confidence level	±1.1
Standard deviation	0.95
Coefficient of variance (%)	1.02

The t-test and f-test were applied to compare 2 equivalent samples for which 5 single readings were taken for each sample. The f- and t- test analyses are presented in Figure 4.3 and Appendix C2. It can be concluded that the differences between the variances and means are not significant. For the means and variances respectively, the one-tail probabilities are 0.206 and 0.383. The t-test used assumed the replicates had unequal variances. Since the probabilities obtained in both cases were much greater than 0.1, this implies that there is no significant difference between the means and variances of these two samples.

F-Test: Two-Sample for Variances

	Sample 1	Sample 2
Mean	93.68	93.07
Variance	0.91	1.21
Observations	5	4
df	4	3
F	1.32	
P(F<=f) one-tail	0.384	
F Critical one-tail	6.59	

t-Test: Two-Sample Assuming Unequal Variances

	Sample 1	Sample 2
Mean	93.68	93.07
Variance	0.91	1.21
Observations	5	4
Hypothesized Mean Difference	0	
df	6	
t Stat	0.880	
P(T<=t) one-tail	0.206	
t Critical one-tail	1.94	
P(T<=t) two-tail	0.413	
t Critical two-tail	2.45	

Figure 4.3: t-test and F-test for the viabilities of two equivalent samples

4.4.2 PROTEASE

The protease assay gave a relatively higher coefficient of variance as compared with that recorded in literature. Robinson (2001) found the coefficient of variance for samples with an average protease absorbance of 0.6 to be 4.1%. The standard deviation calculated in this study from 4 samples of fresh yeast was 0.001 resulting in a coefficient of variance of 3.7% and 4.2% for 2 replicates (Table 4.2). The confidence limit of the protease absorbance at 95% confidence level was calculated to be 0.002 (Table 4.2). This value refers to the statistically allowable limit (difference between any two protease absorbance readings). Thus, if this difference is greater than 0.002, it would be considered to be statistically significant.

The t-test assuming equal variances was used for these replicates and it was concluded that the means are only 60.0% significant (one-tail) implying insignificant difference (Appendix C2). The same conclusion of insignificant difference can be made for the variances, as they were only 58.4% significant.

Table 4.2: Protease statistics

Sample No.	Protease Absorbance	
	Experiment 1	Experiment 2
1	0.034	0.035
2	0.034	0.032
3	0.033	0.034
4	0.036	0.035
Average	0.034	0.034
Standard Deviation	0.001	0.001
Confidence limits at 90% confidence level	±0.002	±0.002
Coefficient of variance (%)	3.7	4.2

4.4.3 VITALITY

The statistical significance gained from the assays used in determining the state of metabolic activity of the yeast, using the Excel analysis toolkit is given in Section 4.3.3.1. The reproducibility of the vitality experiments is explained in Section 4.3.3.2.

4.4.3.1 Assays

Five single-time replicate samples were analysed for glucose concentration and the results obtained are recorded in Table 4.3. The coefficient of variance for this method was found to be 1.7%. The substrate utilisation curve in Figure 4.4 confirms the reproducibility of the DNS method for glucose assay. In the case of biomass concentrations, the coefficient of variance is 4.2% with a standard deviation of 0.08×10^6 cells/ml (Table 4.3). The confidence limits (95% confidence level) for glucose concentration and biomass concentration are 0.01 g/l and 0.12×10^6 cells/ml.

Table 4.3: Vitality statistics

Sample No.	Glucose concentration (g/l)	Biomass concentration (10^6 cells/ml)
1	0.64	1.83
2	0.64	1.72
3	0.62	1.88
4	0.62	1.88
5	0.63	
Average	0.63	1.83
Standard Deviation	0.01	0.08
Confidence limits at 95% confidence level	±0.01	±0.12
Coefficient of variance (%)	1.7	4.2

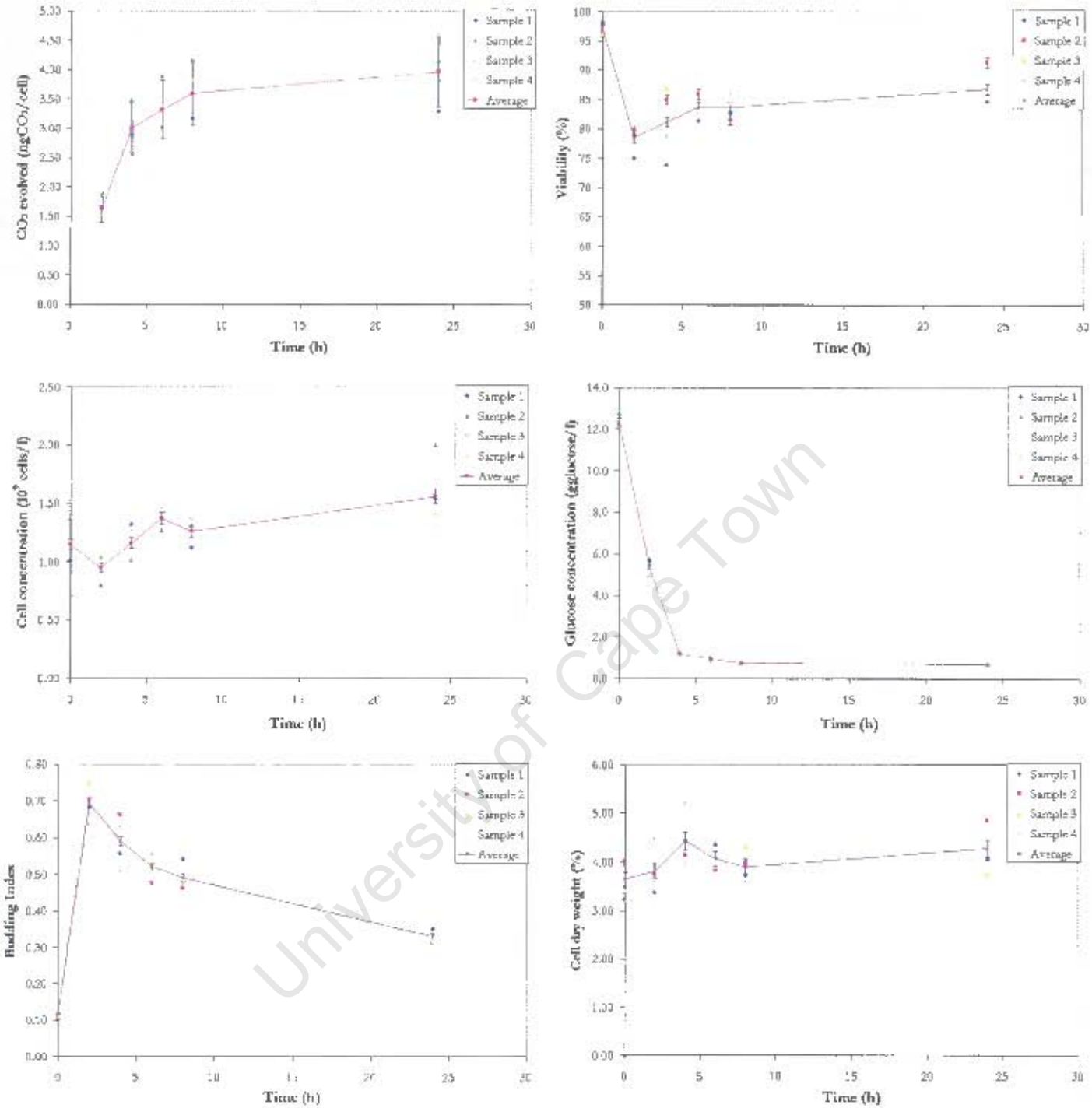


Figure 4.4: Reproducibility of small-scale fermentation

4.4.3.2 Reproducibility of small-scale fermentation experiments

The growth, substrate utilisation, viability, budding indices and carbon dioxide formation and cell dry weight curves obtained from 4 small-scale fermentation replicates were compared in Figure 4.4. The ANOVA was used to conclude whether there is a difference between the replicative samples. A summary of the probabilities obtained are shown in Table 4.4 and of all

the results, carbon dioxide formation and the viability data are not reproducible at a 90% confidence level. The full ANOVA table is recorded in Appendix C3.

Table 4.4: ANOVA for small-scale fermentation

Parameter	Probability		Confidence Level (%)	
	Time	*Sample*	*Time*	*Sample*
Viability	$2.91*10^{-6}$	0.0987	>99.99	90.13
Biomass concentration	0.0037	0.5003	99.63	49.97
Cell dry weight	0.1024	0.2081	89.76	79.19
Glucose concentration	$1.11*10^{-21}$	0.3637	>99.99	63.63
Budding Index	$1.66*10^{-10}$	0.9341	>99.99	6.59
Carbon dioxide formation	$3.37*10^{-7}$	0.0025	>99.99	99.75

It can be concluded from Table 4.4 that the difference in the viability and carbon dioxide formation data are significant at 90% confidence level. Consequently, these sets of data will not be used in analysing the small-scale fermentation data. However, the time profiles are more than 99.0% significant which is expected. This significance is as a result of cell growth.

4.4.3.3 Error in Growth calculations

Table 4.5 is a summary of coefficient of variance, standard deviation and average for the 4 replicate small-scale fermentation experiments. These values have been calculated for growth rates, biomass yield, carbon dioxide yield, substrate utilisation rate, carbon dioxide formation rate and change in cell concentration.

Table 4.5 is consistent with the result obtained in Section 4.3.3.2 for carbon dioxide formation rates. In Table 4.5, the coefficient of variance for the carbon dioxide formation rates is the highest, almost 40%. The high coefficient of variance for the carbon dioxide formation rates establishes that the carbon dioxide data can not be used. Next to the high variations in carbon dioxide formation rates are the substrate utilisation rates with a coefficient of variance of 17.7%.

Table 4.5: Reproducibility of vitality calculations

Sample No.	μ_{max}	X_f/X_o	$Y_{X/S}$		$Y_{CO_2/S}$		q_S		q_{CO_2}	
			(10^9 cells/gglucose)		($ngCO_2$/cell.ggluose)		(g/l.hr)		($ngCO_2$/cell.hr)	
	(hr^{-1})		average	exponential	average	exponential	average	exponential	average	exponential
1	0.110 (0.922)	2.58	0.45	1.37	1.00	3.75	-0.15	-0.09	0.17	0.10
2	0.110 (0.999)	2.37	0.38	1.54	1.06	4.35	-0.13	-0.13	0.18	0.25
3	0.106 (0.963)	2.51	0.39	1.37	1.19	4.52	-0.16	-0.14	0.20	0.11
4	0.086 (0.997)	2.11	0.39	1.44	0.90	3.27	-0.12	-0.12	0.16	0.05
Average	0.103	2.39	0.43	1.43	1.04	3.97	-0.14	-0.12	0.18	0.08
Standard Deviation	0.011	0.21	0.03	0.08	0.12	0.58	0.02	0.02	0.02	0.03
Coefficient of variance (%)	10.8	8.7	8.2	5.8	11.6	14.5	12.1	17.7	11.6	38.5

4.4.4 HAZE ANALYSIS

To interrogate the haze analysis, two yeast suspensions were used. An untreated yeast suspension with a wet weight of 55.3% was analysed in quadruplicate (Figure 4.5a, Figure 4.6a). A yeast suspension with concentration of 54.4% based on wet weight and 14.3% based on dry weight that had been subjected to shear stress at an impeller speed of 400rpm with a Rushton turbine was analysed in duplicate (Figure 4.5b, Figure 4.6b). Figure 4.5 gives the particle size analysis of the range 0 to 50 µm while, Figure 4.4 homes into the region in which haze is prevalent i.e. 0 to 2 µm. An analysis of these particle size distributions in terms of average particle size and ratio of haze material to total material (A_2/A_1) are presented in Table 4.6.

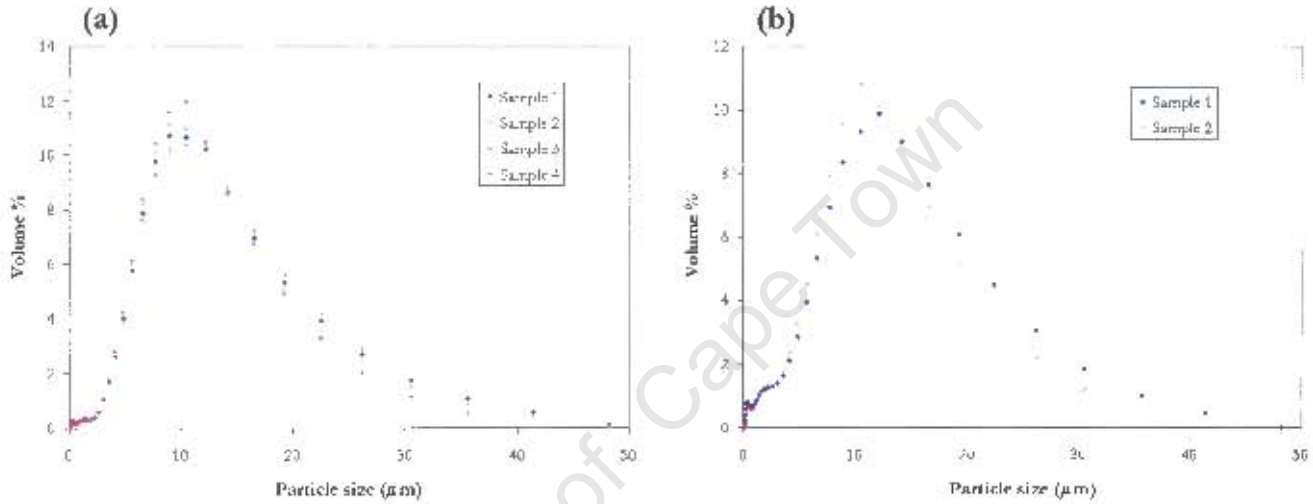


Figure 4.5: Particle size distribution of yeast suspension (complete range)
(a) – Untreated yeast suspension; suspension concentration of 55.3% based on wet weight
(b) – Treated yeast:: 54.4% wet weight; Impeller: pitched blade, 800rpm; Temperature: 4°C

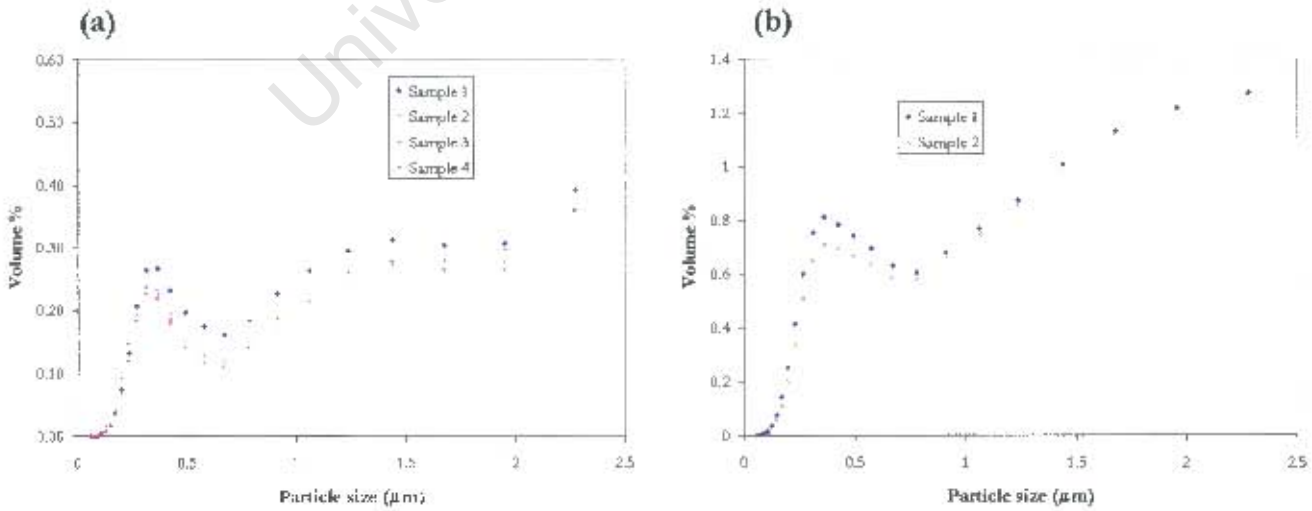


Figure 4.6: Particle size distribution of untreated and treated yeast (sizes < 2µm)
(a) – Untreated yeast suspension; suspension concentration of 55.3% based on wet weight
(b) – Treated yeast: 54.4% wet weight, Impeller: pitched blade, 400rpm; Temperature: 4°C

Table 4.6: Reproducibility of Haze analysis

Sample No.	Average Particle size (X_{50}) (μm)		A_2/A_T (%)	
Yeast Treatment	Untreated	Treated	Untreated	Treated
1	9.24	9.42	0.16	0.54
2	9.13	8.88	0.14	0.55
3	9.48		0.12	
4	8.96		0.14	
Average	9.20	9.15	0.14	0.55
Standard Deviation	0.22	0.38	0.02	0.09
Coefficient of variance (%)	2.4	4.2	13.8	4.8

The two-way ANOVA was used to analyse the particle size distribution data and it was concluded that there is no significant difference between the 4 untreated samples or between the 2 treated samples despite the fact that the sample size is small (2 samples as opposed to 4 samples) at a 90% confidence level (Appendix C4). The particle size distribution method is therefore reproducible. Difference between the haze present before and after shear stress is clearly seen.

4.5 REPRODUCIBILITY OF MIXING EXPERIMENTS

The reproducibility of the mixing data depends on a number of operating variables such as: cell concentration, impeller speed or extent of agitation, tracer amount, air entrainment and injection port. To illustrate how the cell concentration affects the mixing time, Figures 4.7, 4.8 and 4.9 are shown. In Figure 4.7, the yeast suspension used has a concentration of 41.9% wet weight. It can be clearly seen that the data is reproducibility although by the sixth addition, the tracer concentration is high and the data becomes less reproducible. It is possible that the difference in salt concentrations causes osmotic stress to the yeast. As the suspension concentration increases first to 52.3% wet weight (Figure 4.8) and later to 62.8% wet weight, the mixing data becomes less reproducible. Generally in these cases, the solution only reached 16.7% homogeneity after 10 minutes of mixing when the yeast concentration is 52.3% wet weight (Figure 4.8) and less than 10.0% homogeneity at 62.8% wet weight. Further, the 1st sample tends to attain some form of homogeneity whilst subsequent additions give negative changes in conductivity. Thus, it can be concluded that the more the number of subsequent additions and the higher the suspension concentration, the less reproducible the mixing data become.

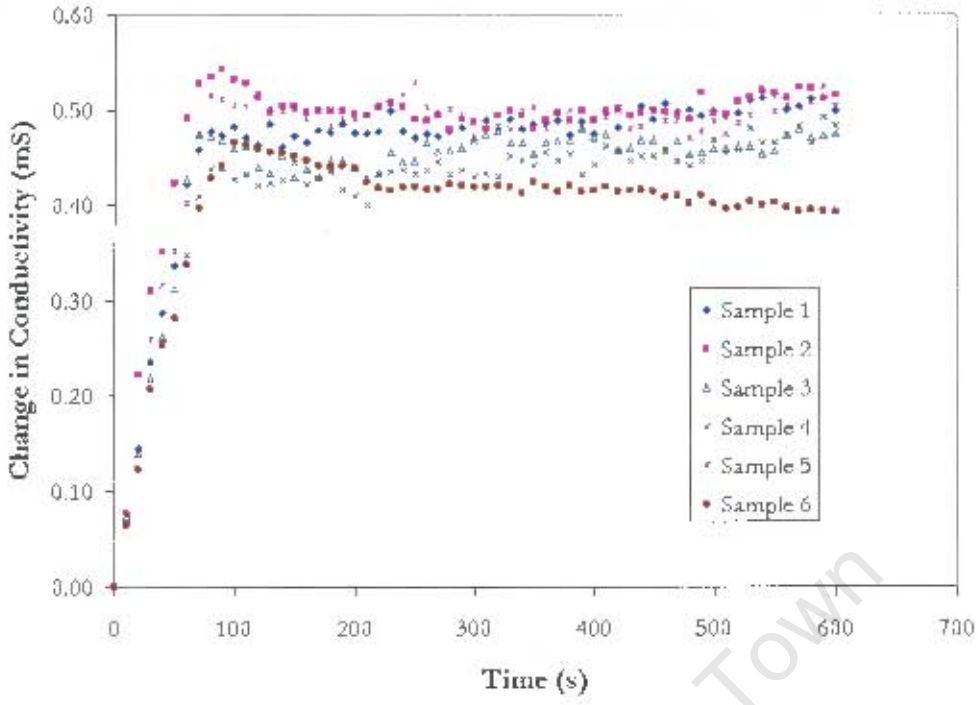


Figure 4.7: Reproducibility of replicative mixing data at 41.9% wet weight.
Suspension concentration: 41.9% wet weight. Impeller: Rushton turbine at 400 rpm.
Temperature: 14°C

Samples 1 to 6 represent data collected following each of 6 sequential additions of tracer to the same yeast suspension

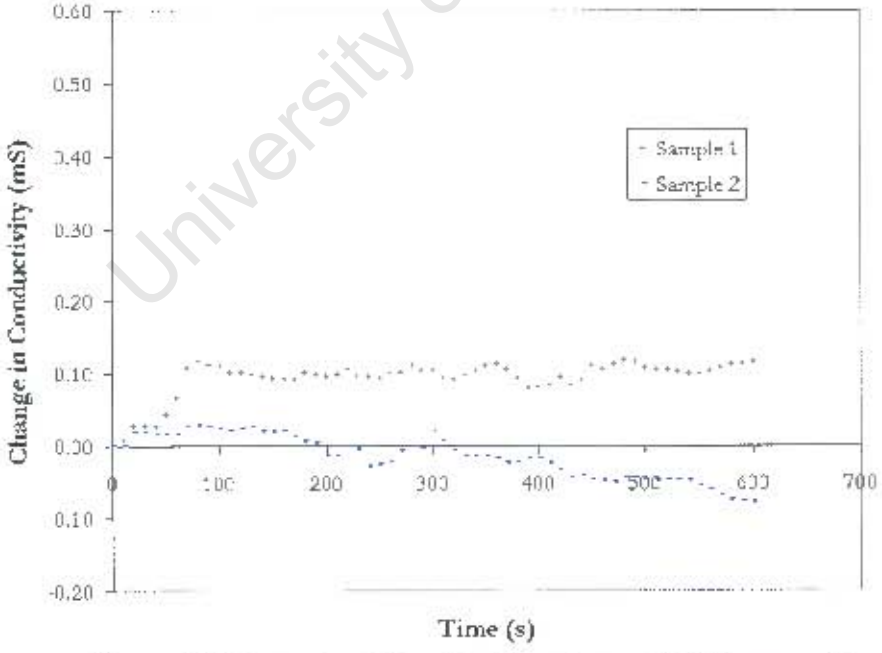


Figure 4.8: Reproducibility of mixing data at 52.3% wet weight.
Suspension concentration: 52.3% wet weight. Impeller: Rushton turbine at 400 rpm.
Temperature: 14°C

Samples 1 and 2 represent data collected following each of 2 sequential additions of tracer to the same yeast suspension

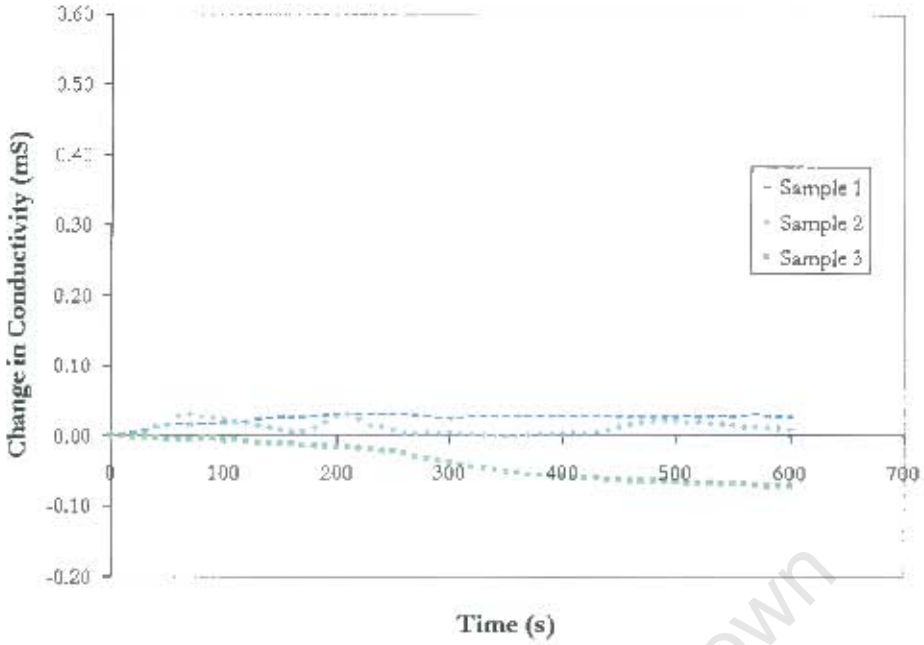


Figure 4.9: Reproducibility of mixing data at 62.8% wet weight.
Suspension concentration: 62.8% wet weight. Impeller: Rushton turbine at 400 rpm.
Temperature: 14°C
Samples 1 to 3 represent data collected following each of 3 sequential additions of tracer to the same yeast suspension

The two-way ANOVA was used to determine the variations in 3 replicates of mixing experiments conducted using yeast suspension with concentration of 36.1% wet weight and Rushton turbine at an agitation rate of 800 rpm at 14°C. The data are reproducible with probability of 0.477 (Appendix C5). Data obtained using a nitrogen blanket were not significantly different at a 90% confidence level from those obtained with the reactor open to the atmosphere (Appendix C6). The probability obtained in this case was 0.168 implying that the difference is only significant at 84.2% confidence level.

The mixing data obtained from 5 replicates determined using the Rushton turbine at a speed of 300rpm and a yeast concentration of 23.4% wet weight shown in Figure 4.10 are recorded in Table 4.7. The coefficient of variance of the final 40 data points did not exceed 5.0%.

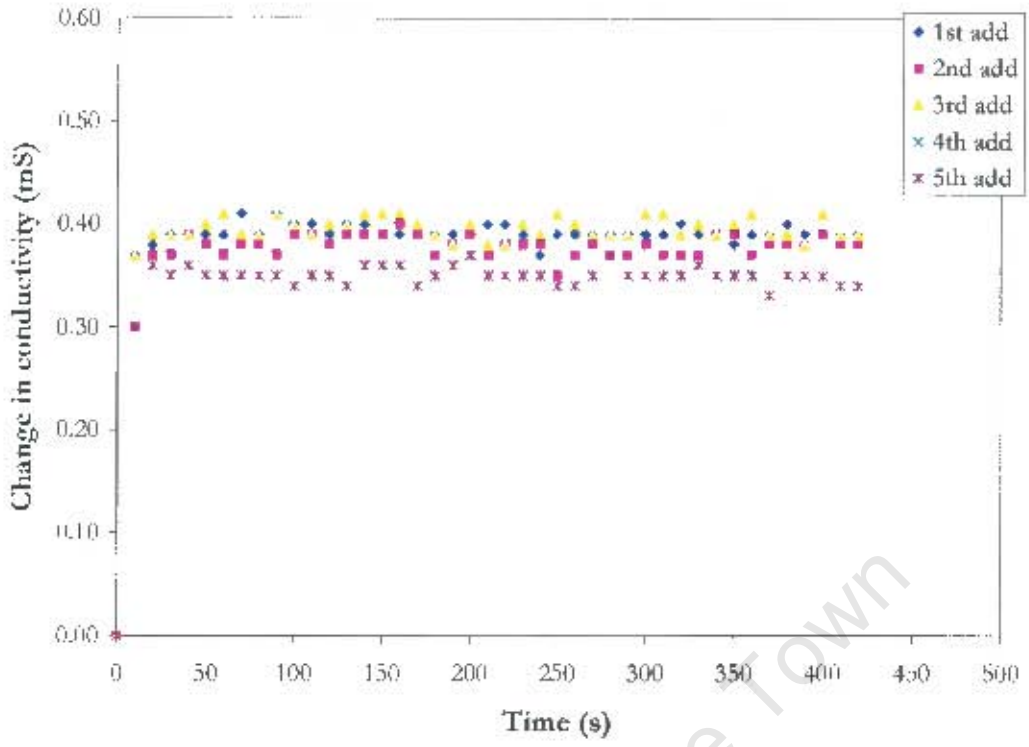


Figure 4.10: Reproducibility of mixing time, t_m.
Suspension concentration: 23.4% wet weight. Impeller: Rushton turbine at 300 rpm.
Temperature: 14°C
Samples 1 to 5 represent data collected following each of 5 sequential additions of tracer to the same yeast suspension

Table 4.7: Coefficient of variance and standard deviation for mixing time obtained from the data in Figure 4.8

Sample No.	Mixing time, t_m (s)	
	t_{67}	t_{95}
1	7	12
2	8	18
3	7	13
4	7	11
5	8	15
Average	7.48	13.9
Confidence limit at 95% confidence level	0.68	3.45
Standard Deviation	0.59	3.00
Coefficient of variance	7.89	21.6

4.6 Discussion And Conclusions

The t-test, f-test and ANOVA were the statistical tools used to analyse the results obtained from the investigation into the effect of agitation on yeast quality. The t-test was used to check the significance of the difference between two means while the f-test was used for comparing two variances. The f-test is a pre-requisite to the t-test because one of the requirements for the validity of the t-test is that the variances of the population being tested are not significantly different. Conversely, the ANOVA is used to gauge the significance of several means.

Yeast suspension collected on different days were tested for significance by the use of the ANOVA and it was concluded that the there is no significant difference in the time profile of the viabilities of the yeast suspension collected on different days. Furthermore, there were no observed variations in the viability/time profiles of yeast suspensions of different generations.

The coefficients of variance for the different analytical methods were calculated. For the methylene blue staining method, it was found to be 1.1%. In addition, the differences between the variances and means of two replicates of 5 single methylene blue staining samples were found to be insignificant. For the protease assay, coefficient of variance was about 4.0% with there being no significant difference in the variances and means of 2 replicates of 4 single samples. The differences between size distribution curves for the amount of haze found in treated and untreated yeast were found to be insignificant. Further, the coefficients of variance for the amount of haze were 13.8% for untreated yeast and 4.8% for treated yeast.

Vitality was quantified by performing small-scale fermentations and monitoring the growth rates, glucose utilisation rates, product formation rates, viabilities and budding indices over a period of 24 hours. For glucose assay and biomass concentrations, the coefficients of variance were 1.7% and 4.2% respectively. In addition, the time profiles for 4 replicate small-scale fermentations were tested with ANOVA and the viability and carbon dioxide formation data were found to be significant at a 90.0% confidence level. Consequently, the carbon dioxide formation and viability data are not reliable. The coefficients of variance for some of the growth parameters such as carbon dioxide formation rates and growth rates were quite high (> 20%).

Finally, the mixing data were found to be less reproducible the higher the cell concentration and the more the number of tracer additions. In comparing the data obtained from experiments conducted with a nitrogen blanket and those obtained without, there is no significant difference between these sets of data. This shows that as long as the injection points are the same and if the flow rate of the nitrogen gas is moderate, the mixing data would be reproducible. The coefficients of variance for mixing times at 67% and 95% homogeneity were found to be 7.89% and 21.6% respectively.

CHAPTER 5: RHEOLOGY AND MIXING

5.1 INTRODUCTION

The methods by which the degree of mixing in the vessel and rheology of the yeast suspension were determined are reported in Chapter 3. On characterizing the rheology of the yeast suspension the apparent viscosity was determined as a function of shear rate, which consequently helps to determine the nature of the fluid, Reynolds Number and the power dissipated per unit volume under the range of mixing conditions. Reynolds number is important in determining the type of flow in the vessel and power dissipated per unit volume relates to the energy consumption of the impellers. Mixing time gives an indication of the degree of mixing in a vessel.

Chapter 5 focuses on the rheology of the yeast suspension and the degree of mixing attained at different concentrations and agitation intensities. In Section 5.2, the rheogram and rheology parameters are discussed while Section 5.3 focuses on the degree of mixing. In Section 5.3, the mixing times are reported for 66.7% and 95.0% homogeneity of the final conductivity.

5.2 YEAST RHEOLOGY

The rheology of the fluids was determined by using the VT520 Haake concentric cylinder viscometer to measure the apparent viscosity (μ) as a function of shear rate (γ) and obtain a rheogram. Rheograms were obtained for yeast suspension concentrations between 10 and 60% wet weight (1.5 and 13.3% dry weight) as well as 2% CMC solution and are presented in Figure 5.1. From the data presented in Figure 5.1, it is apparent that the yeast suspension is a pseudo-plastic fluid (i.e. the apparent viscosity decreases with increasing shear stress) between the concentration ranges of 20.0 to 66.3% (based on wet weight). As the cell concentration reduced below 20%, the rheology approximated as a Newtonian fluid i.e. the viscosity is independent of the shear rate. In the higher concentration ranges (58.3 to 66.3%), the yeast suspension had yield stresses that increased as the suspension concentration increased. At the yeast suspension concentration of 58.3%, the value of the yield stress was 14 Pa and 20 Pa at a suspension concentration of 66.3%. Equation 5.1 which is a power law accurately described the relationship between shear rate and shear stress in the yeast suspension concentration ranges of 20.0 to 44.0% while, Equation 5.2 (Herschel-Bulkley model) describes the relationship in the higher yeast suspension concentration ranges (58.3 to 66.3%). The rheology of the CMC solution is similar to that of the yeast suspension in the intermediate concentration range (between 30.0 and 44.0% wet weight).

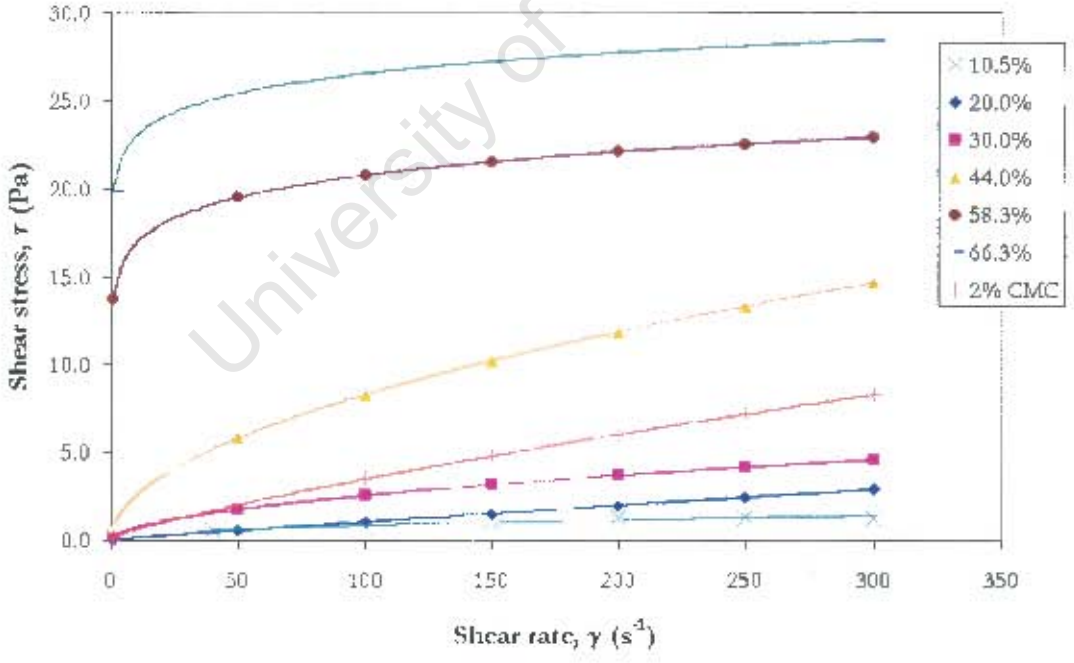


Figure 5.1: Rheograms for cropped yeast suspension with concentrations in the range of 10 to 66% wet weight.

Pseudoplastic fluids can be described by either the power law:

$$\mu = K\,\dot{\gamma}^{n} \text{ ---------- Equation 5.1}$$

or the Herschel-Bulkley model:

$$\mu = \tau_0 + K\,\dot{\gamma}^{n} \text{ ---------- Equation 5.2}$$

where: μ is the viscosity (cP)

K, the consistency index.

$\dot{\gamma}$, the shear rate (s^{-1})

n, the flow behaviour index and

τ_0, the yield stress (Pa)

Figure 5.2 shows the rheology parameters n and K obtained graphically from the plot of ln μ against ln $\dot{\gamma}$. These parameters were defined in Equation 5.1 and 5.2. The R^2 values obtained from the linear regression are shown in Table 5.1. The linear regression obtained for the yeast suspension in the lower concentration range of 10.5% wet weight had a low R^2 value suggesting that the level of uncertainty in the values of K and n has given by the power law fit is higher. The consistency index increased while the flow behaviour index decreased as the yeast suspension concentration increased. The flow behaviour index obtained for CMC solution was found to be 0.80 which is within 2.5% of the literature value (Kouda *et al.*, 1996).

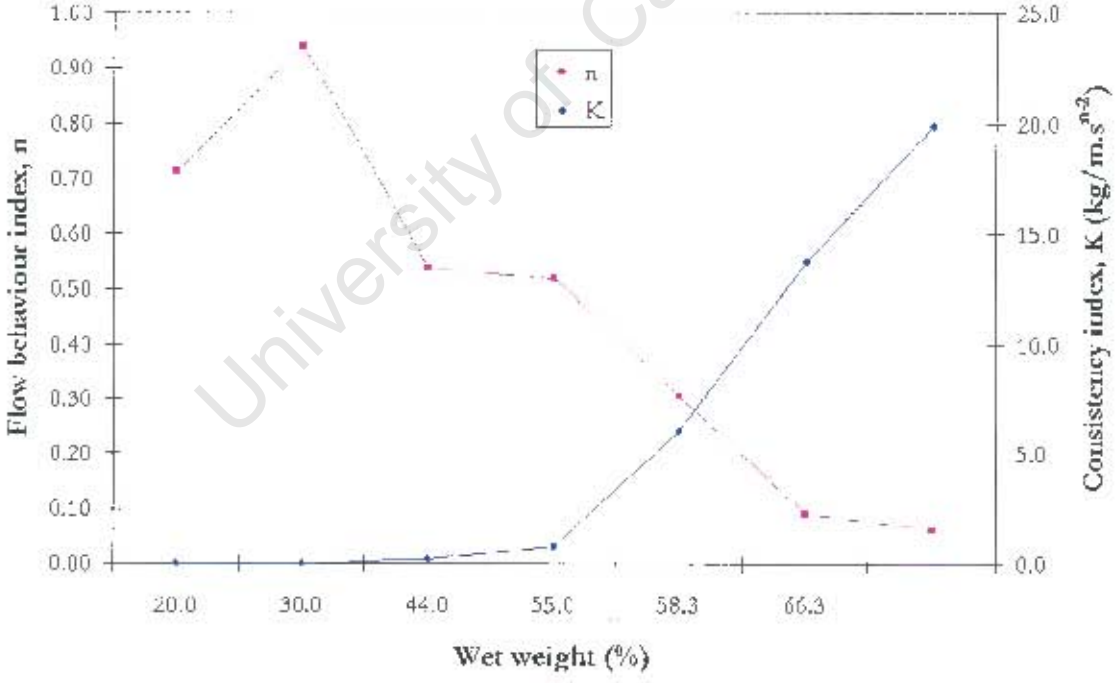


Figure 5.2: Rheological parameters for different yeast suspension concentrations modelled by the power law.

Pseudoplastic fluids can be described by either the power law:

$$\mu = K \gamma^n \text{ ---------- Equation 5.1}$$

or the Herschel Bulkley model:

$$\mu = \tau_o + K \gamma^n \text{ ---------- Equation 5.2}$$

where: μ is the viscosity (cP)

K, the consistency index.

γ, the shear rate (s^{-1})

n, the flow behaviour index and

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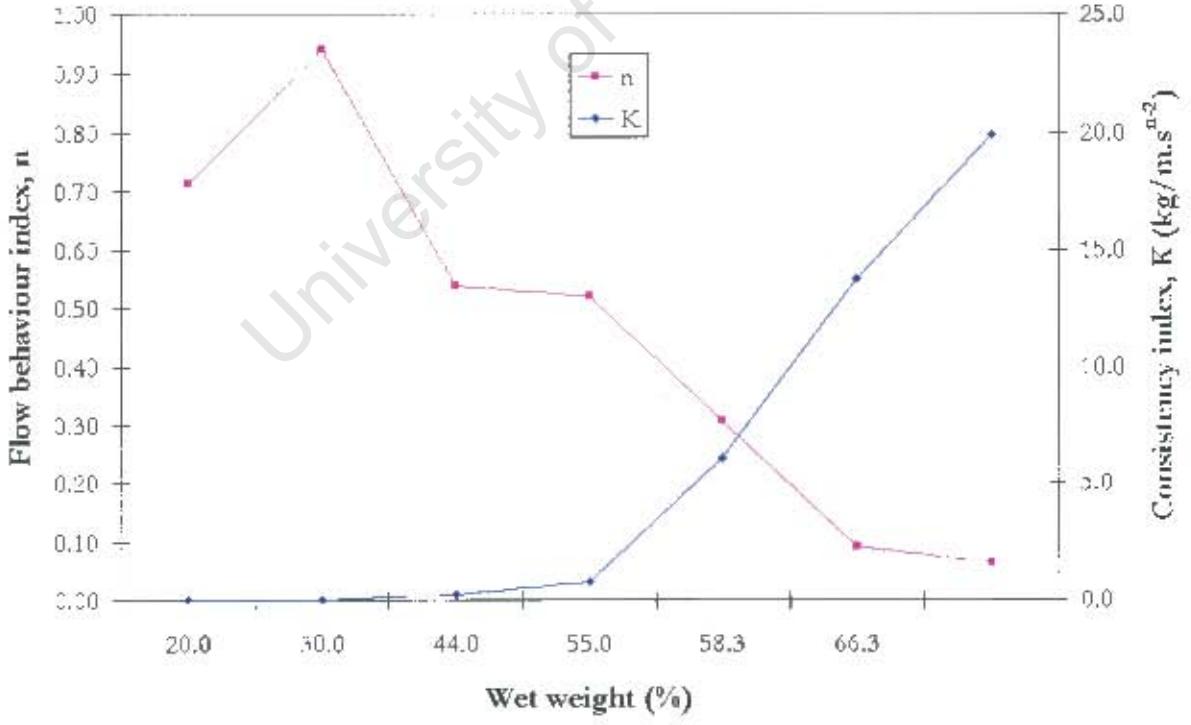

Figure 5.2: Rheological parameters for different yeast suspension concentrations modelled by the power law.

Table 5.1: R^2 values for the rheological parameters in Figure 5.2

Wet weight (%)	τ_o (Pa)	n	K (kg/m.s^{n-2})	R^2
10.5	—	0.713	0.027	0.7040
20.0	—	0.941	0.013	0.9380
30.0	—	0.539	0.209	0.9998
44.0	—	0.521	0.752	0.9967
58.3	14.0	0.090	13.8	0.9998
66.3	20.0	0.063	19.9	0.9981
2% CMC	—	0.798	0.09	0.9958

In Table 5.2, the equations obtained from fitting the power and exponential laws to the consistency index and flow behaviour index as a function of the yeast suspension concentration are shown. The exponential law gives a better fit than the power law with respect to the R^2 values.

Table 5.2: Rheological parameters K and n as a function of suspension concentration

Parameter	Power law		Exponential law	
	Equation	R^2	Equation	R^2
K	$4*10^{-7}x^{4.11}$	0.859	$0.003e^{0.138x}$	0.954
N	$621x^{-2.07}$	0.776	$3.38e^{-0.056x}$	0.853

In Table 5.2: x = yeast suspension concentration based in wet weight (%)

In Figure 5.3, the relationship between yeast concentration (based on wet weight) and apparent viscosities at typical YCV and experimental set-up agitation rates are shown. The apparent viscosities were calculated using the consistency index and flow behaviour index shown in Table 5.1. The agitation rates typically used in the YCV are 60 and 100 rpm which are equivalent to shear rates of 13.4 and 22.3 s^{-1} and those used in the experimental stirred tank reactor (STR) were 200 and 800 rpm (equivalent to shear rates of 44.6 and 178 s^{-1}). Generally, there is a marked increase in the apparent viscosities as the yeast suspension concentration increase. In comparing the range of agitation rates, initially, at low cell concentrations (less than 30% wet weight), the apparent viscosities are similar. However at 66.3% wet weight, the apparent viscosity at an agitation rate of 60 rpm is 1.6 times that at 100 rpm and 11.3 times that at 800 rpm.

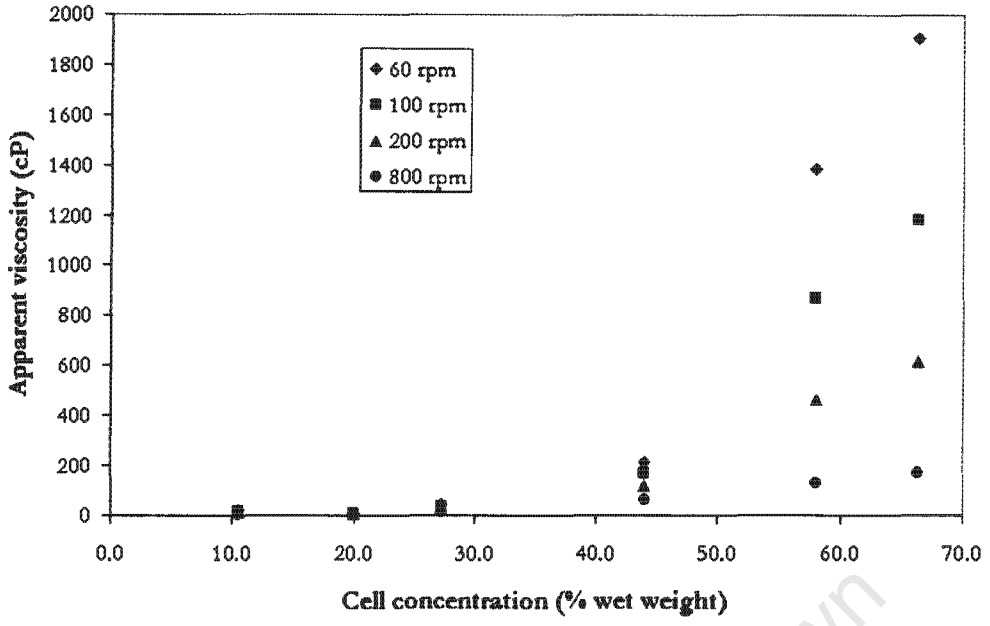

Figure 5.3: Apparent viscosities at typical agitation rates of the YCV and experimental STR.

In order to be able to predict the apparent viscosities at different cell concentrations, the curves obtained in Figure 5.3 were modelled using exponential, power law and linear fits. Equation 5.3 refers to the general equation of an exponential curve, Equation 5.4 to that of a power law model and Equation 5.5 to that of a line. Other models used were the Einstein equation shown in Equation 5.6 and the Vand equation shown in Equation 5.7 (Reuss *et al.*, 1979).

$$y = ae^{bx} \quad \text{---------- Equation 5.3}$$

$$y = ax^{b} \quad \text{---------- Equation 5.4}$$

$$y = ax + b \quad \text{---------- Equation 5.5}$$

$$y = y_s(1 + ax) \quad \text{---------- Equation 5.6}$$

$$y = y_s(1 + ax + bx^2) \quad \text{---------- Equation 5.7}$$

where: y is the apparent viscosity (cP)

x, the yeast suspension concentration based on wet weight (%)

a and b are model parameters

y_s is the viscosity of the supernatant (cP)

In Table 5.3, it is seen that the fits as shown by the R^2 values improve as the shear and agitation rates increase. However, in the entire range of shear and agitation rates being investigated, the exponential and the Vand equation models give the better fits (as shown by the R^2 values).

Table 5.3: Model parameters for the relationship between apparent viscosity and yeast concentration at the agitation rates used in the YCV and experimental STR

Shear rate (Agitation rate)	13.4 s^{-1} (60 rpm)				22.3 s^{-1} (100 rpm)			
Model Parameters	y_s (cP)	a	b	R^2	y_s (cP)	a	b	R^2
Exponential fit	-----	3.06	0.100	0.967	-----	3.08	0.092	0.973
Power law fit	-----	0.004	3.00	0.884	-----	0.007	2.78	0.897
Linear fit	-----	34.0	-684	0.819	-----	21.1	-416	0.835
Einstein equation	-684	-20.1	-----	0.819	-416	-19.7	-----	0.835
Vand equation	468	-0.101	0.002	0.978	263	-0.102	0.002	0.982
Shear rate (Agitation rate)	**44.6 s^{-1} (200 rpm)**				**178 s^{-1} (800 rpm)**			
Model Parameters	y_s (cP)	a	b	R^2	y_s (cP)	a	b	R^2
Exponential fit	-----	3.12	0.082	0.981	-----	3.18	0.063	0.989
Power law fit	-----	0.013	2.50	0.915	-----	0.043	1.93	0.955
Linear fit	-----	11.1	-209	0.863	-----	3.01	-47.6	0.935
Einstein equation	-209	-18.8	-----	0.863	-47.6	-15.8	-----	0.935
Vand equation	114	-0.103	0.003	0.988	13	-0.099	0.004	0.999

Table 5.4 shows the Vand equation and exponential model parameters for the complete data set. Finally, Table 5.5 shows the errors obtained from using the Vand and exponential equations to calculate the apparent viscosities in the yeast suspension concentration range used in this study. Also, Table 5.5 shows that the Vand equation gives a better prediction in the higher concentration ranges while, the exponential equation is more accurate in the lower to medium concentration ranges. Another point to be noted in the use of the Vand equation is that the minimum point is at 21.7 % wet weight (-21.7 cP) and apparent viscosity-axis intercepts are 15.1 and 28.2 % wet weight. This means that the apparent viscosity is negative between 15.1 and 28.2 % wet weight hence, not physically possible.

Table 5.4: Vand equation and exponential model parameters for the relationship between apparent viscosity and yeast concentration for the complete data set

Model Parameters	y_s (cP)	A	b	R^2
Exponential fit	-----	2.87	0.090	0.978
Vand equation	214	-0.102	0.002	0.982

Table 5.5: Error values of the Vand and exponential equations for yeast suspensions.

Wet weight (%)	Actual value (cP)	Exponential equation		Vand equation	
		Prediction (cP)	Error (%)	Prediction (cP)	Error (%)
10.4	9.54	7.4	22.4	40.9	329
20.0	10.6	17.4	63.7	-20.4	291
27.4	41.6	33.6	19.2	-5.71	114
44.0	141	151	7.50	230	63.0
58.3	712	549	22.8	654	8.02
66.3	967	1129	16.8	982	1.53

5.3 MIXING OF CROPPED YEAST SUSPENSION IN THE STIRRED TANK REACTOR (STR)

The degree of mixing of the stirred tank was determined by the use of an aliquot of 2 ml of 3M NaCl tracer injected into 910 ml of the agitated yeast suspension. The conductivity of the resultant mixture was monitored with time. Two mixing times have been defined, t_{67} and t_{95} which are the times taken for the conductivity readings to reach 67% and 95% of the final homogeneous conductivity. The coefficient of variance for t_{67} is 7.89% and that for t_{95} is 21.6%. The method by which these coefficients of variance were determined and its application are shown in Sections 3.5 and 4.4.

5.3.1 CHANGE IN MIXING TIME WITH IMPELLER SPEED

Figures 5.4 and 5.5 show the change in mixing time with concentration for agitation rates of 200, 400, 600 and 800 rpm equivalent to impeller tip speeds of 0.73 to 2.93 ms^{-1}. The Rushton turbine of standard geometry (See Section 3.7 for the dimensions) was used in this case. In Figure 5.4 t_{67} has been plotted whilst in Figure 5.5, t_{95} is used. It is apparent from the data presented that the mixing time decreases with increasing speed. At a suspension concentration of 43.1% wet weight, there is a 10-fold decrease in the mixing time as the impeller tip speed increases by 4 fold. This decreases to a 6-fold decrease in the mixing times as the agitation rate increases from 200 to 800 rpm at a suspension concentration of 37.8%. At a wet weight suspension concentration of 31.7% wet weight, agitation rate has little observable effect on mixing time.

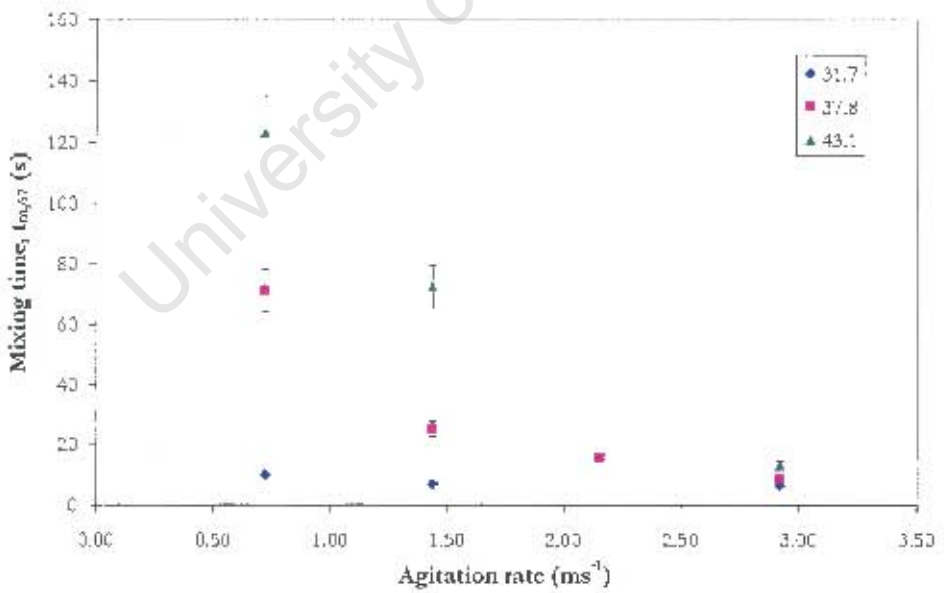

Figure 5.4: Effect of agitation rate and yeast concentration on mixing time, t_{m67} using the Rushton turbine at 14°C

Legend refers to the wet weight % of yeast suspension

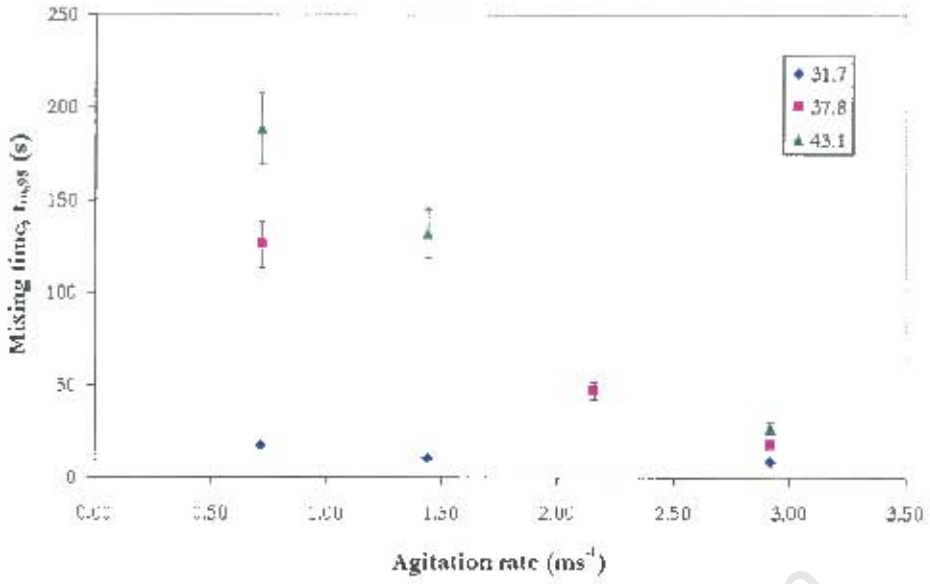

Figure 5.5: Effect of agitation rate and yeast concentration on mixing time, $t_{m,95}$, using the Rushton turbine at 14°C

Legend refers to wet weight % of yeast suspension

The curves obtained in Figures 5.4 and 5.5 were fitted with exponential, power law and logarithmic models and the model parameters have been reported as a and b in Table 5.6. Of the three models, the power law and exponential models present a good prediction of the mixing time/yeast concentration relationship. And of these two models, the power law gives a better fit to the experimental data than the exponential model at yeast concentrations of 31.7% and 37.8%. However, at a yeast concentration of 43.1% based on wet weight, the exponential model gives a better fit.

Table 5.6: Modelling mixing time as a function of agitation rates at 14 °C

Mixing time, $t_{m,67}$									
Yeast concentration (Wet weight %)	**31.7**			**37.8**			43.1		
Model Parameters	a	B	R^2	a	b	R^2	a	b	R^2
Exponential model	10.3	-0.184	0.763	97.2	-0.957	0.917	303	-0.992	0.980
Power law model	8.48	-0.323	0.902	35.9	-1.57	0.991	99.6	-1.53	0.894
Logarithmic model	2.55	8.62	0.879	-40.2	43.7	0.905	-82.4	108	0.986
Mixing time, $t_{m,95}$									
Yeast concentration (Wet weight %)	**31.7**			**37.8**			**43.1**		
Model Parameters	a	b	R^2	a	b	R^2	a	b	R^2
Exponential model	19.3	-0.304	0.866	481	-1.22	0.993	426	-0.971	0.999
Power law model	14.0	-0.519	0.997	129	-1.90	0.963	145	1.53	0.955
Logarithmic model	-6.33	14.4	0.930	-141	150	0.955	-130	161	0.997

5.3.2 CHANGE IN MIXING TIME WITH CELL CONCENTRATION

Figure 5.6 and 5.7 present the data collected to show the relationship between mixing time and apparent viscosities and cell concentration across a concentration range of 28.9% to 50% wet weight. The Rushton turbine at a speed of 400 rpm (tip speed of 1.46 ms^{-1}) and 800 rpm was used in this set of experiments. The yeast was maintained at a temperature of 14 °C. In Figure 5.6, the mixing t_{67} is presented and Figure 5.7, t_{95} is presented. As expected, the mixing time increases with increasing cell concentration. However, the increase is small in the concentration range of 30 to 40% wet weight and more marked in the range of 40 to 55%. Across the 40 to 55% concentration range, there is a 3 to 6 fold increase in mixing time.

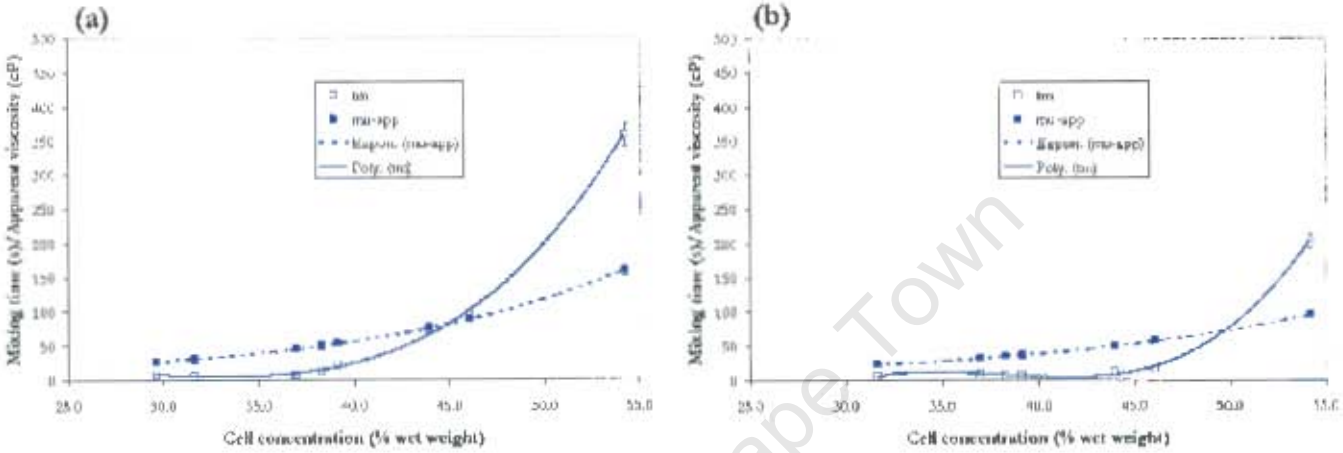

Figure 5.6: Effect of suspension concentration on mixing time ($t_{m,67}$) using Rushton turbine at a vessel temperature of 14°C and agitation rates of: (a) - 400 rpm & (b) - 800 rpm

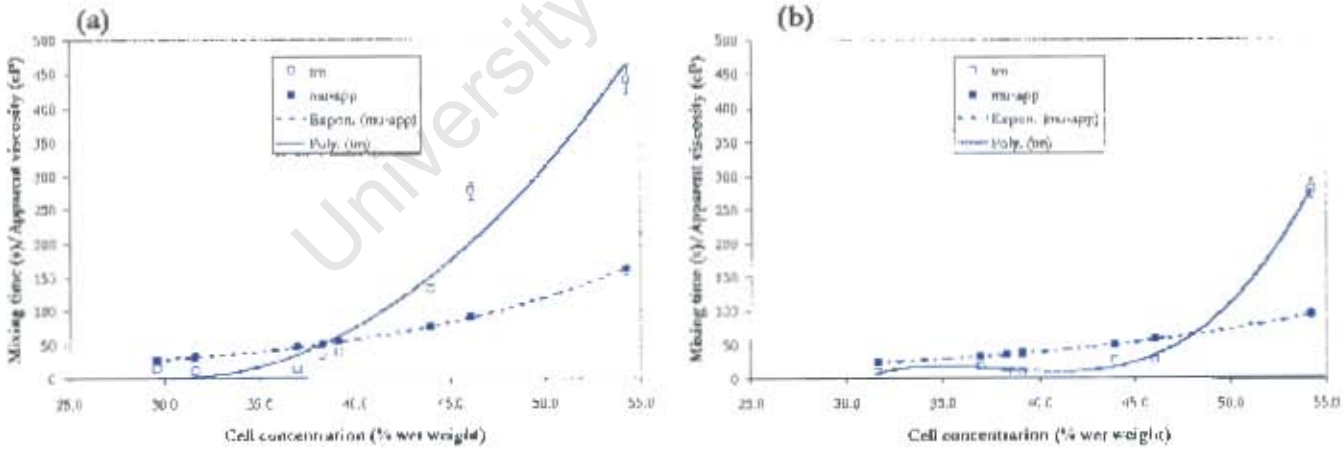

Figure 5.7: Effect of suspension concentration on mixing time ($t_{m,95}$) using Rushton turbine at a vessel temperature of 14°C and agitation rates of: (a) - 400 rpm & (b) - 800 rpm.

Concentrations below 27% have not been included because at these low concentrations, there was gas entrainment, which affected the sensitivity of the conductivity meter. At higher cell concentrations (> 55%) the mixing time exceeded 30 min and was not reproducible (Section 4.4).

In order to be able to accurately predict mixing time at different yeast suspension concentrations, the data obtained in Figures 5.6 and 5.7 were modeled by the use of either an exponential, power law or cubic model. The model parameters are shown in Table 5.7 with a, b, c and d referring to the constants in Equations 5.3, 5.4 and 5.8.

$$y = ax^3 + bx^2 + cx + d \text{ ---------- Equation 5.8}$$

It can be clearly seen in Table 5.7 that the cubic model which had the lowest R^2 value described the relationship between suspension concentration and mixing time the best. The resulting cubic curves are shown in Figures 5.6 and 5.7.

Table 5.7: Modelling mixing time as a function of yeast concentration using Rushton turbine at 14°C

Mixing time, $t_{m,67}$				
Agitation rate (rpm)	400		800	
Models	Equations	R^2	Equations	R^2
Exponential model	$0.024e^{0.175x}$	0.918	$0.022e^{0.154x}$	0.797
Power law	$2*10^{-6}x^{3.94}$	0.881	$1.00*10^{-9}x^{6.29}$	0.722
Cubic	$0.027x^3 - 2.39x^2 + 71.1x - 688$	0.999	$0.054x^3 - 6.21x^2 + 235x - 2920$	0.999
Mixing time, $t_{m,95}$				
Agitation rate (rpm)	400		800	
Models	Equations	R^2	Equations	R^2
Exponential model	$0.055e^{0.172x}$	0.897	$0.034e^{0.155x}$	0.824
Power law	$7.00*10^{-10}x^{5.53}$	0.880	$1.00*10^{-9}x^{6.20}$	0.762
Cubic	$-0.068x^3 + 9.38x^2 - 400x + 5410$	0.978	$0.071x^3 - 8.05x^2 + 304x - 3790$	0.997

The inconsistency of data collected at yeast concentrations above 55% is illustrated in Figure 5.8. Here a typical raw data collected at the highest agitation rate being investigated i.e. 800 rpm (tip speed of 2.93 ms^{-1}) with the Rushton turbine and a yeast suspension concentration of 63.0% are presented. The final change in conductivity should be 0.50 mS/cm. After 600 s, only 20% of the final change has been effected. Not only is mixing slow but the results are not reproducible either as the final change in conductivity for the 1st tracer introduction is 0.05 mS/cm, that of the 2nd one 0.10 mS/cm while, the 3rd introduction gave a final change in conductivity of 0.15 mS/cm.

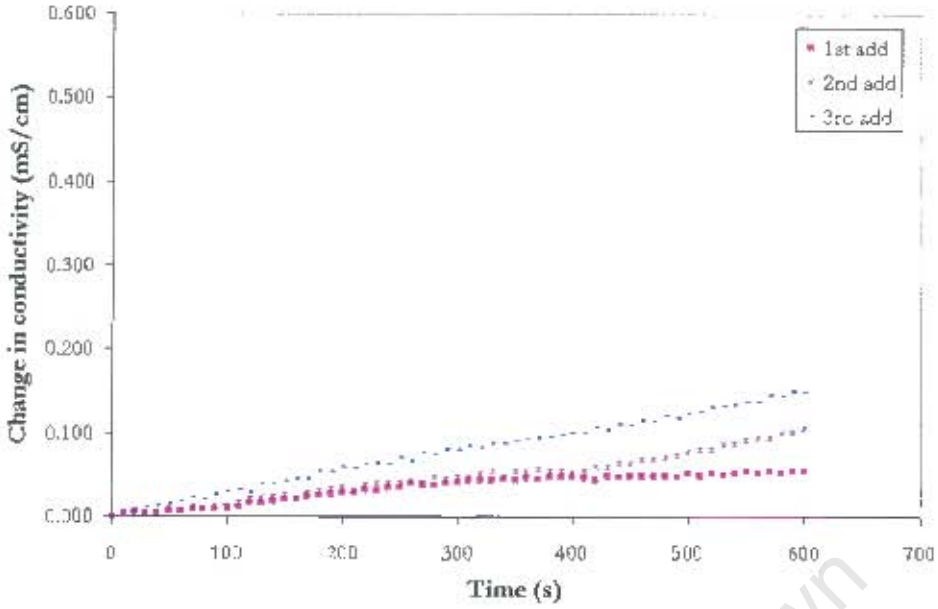

Figure 5.8: Mixing time at 63.0% wet weight using Rushton turbine at an impeller tip speed of 2.93 ms^{-1} and a temperature of 14°C

5.3.3 EFFECT OF IMPELLER TYPE ON MIXING TIME

In Figures 5.9 and Figure 5.10, the effect of impeller type on mixing is investigated. Two impeller types were used for this set of experiments: the Rushton turbine and 45° pitched blade impeller. The range of agitation rates from 200 and 800 rpm was investigated whilst the cooling temperature was maintained at 14°C. Yeast concentrations of 30% and 40% were used. The time taken for the conductivity to reach 66.7% of the final conductivity, t_{67} is shown on the graph in Figure 5.9 and the time taken for the conductivity to reach 95% of the final conductivity, t_{95} is given in Figure 5.10.

The Rushton turbine provided better mixing than pitched blade impeller at both yeast concentrations (30% and 40% based on wet weight) as shown in Figures 5.9 and 5.10. The mixing times for both impellers at an impeller tip speed of 2.93 ms^{-1} (800 rpm) and both suspension concentrations were of the same order of magnitude. However, at the lowest impeller tip speed of 0.73 ms^{-1}, there was a nine-fold increase when comparing the Rushton impeller to the pitched blade at both yeast concentrations. At an agitation rate of 400 rpm (tip speed of 1.46 ms^{-1}) and yeast concentration of 30%, there was a seven-fold increase in $t_{m,67}$ and a nine-fold increase in $t_{m,95}$. Further, at the higher yeast concentration being investigated, there was a four-fold and two-fold increase in $t_{m,67}$ and $t_{m,95}$.

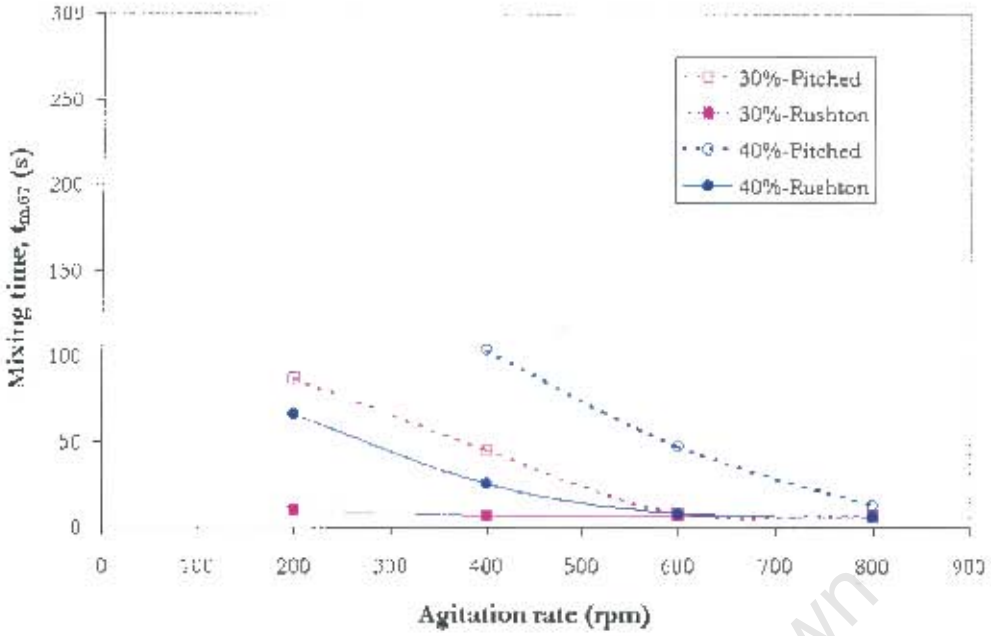

Figure 5.9: Effect of impeller type on mixing at $t_{m,67}$ across an agitation rate range of 200 to 800 rpm and cell concentrations of 30 and 40 % wet weight (given in legend) at 14°C

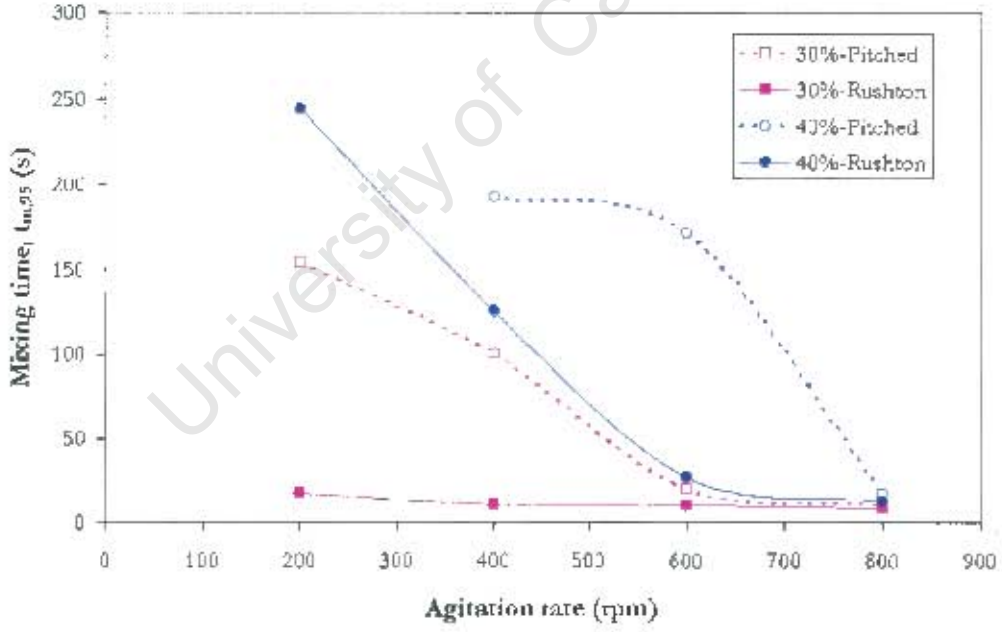

Figure 5.10: Effect of impeller type on mixing at $t_{m,95}$ across an agitation rate range of 200 to 800 rpm and a concentration range of 30 to 47% wet weight (given in legend) at 14°C

5.4 DISCUSSION AND CONCLUSIONS

The factors affecting the achievement of homogeneity in a vessel are: the physical properties of the fluid to be mixed especially its rheology and density; the agitation rate of the impeller; the energy dissipated by the impeller; and the geometry and type of the impeller and vessel used. The vessel and impellers used were of standard geometry (Section 3.7) thereby eliminating the effect of vessel and impeller geometry on mixing. The range of yeast concentrations investigated was between 10.4 and 66.3% wet weight.

In this study, the rheology of yeast suspensions is dependent on the suspension concentration. In the lower concentration ranges (< 20% wet weight), the rheology of the yeast suspension was approximated to be Newtonian. This was shown by the shear stress-shear rate curves obtained for the suspension concentrations at 10.5% and 20% wet weight in Figure 5.1. These curves which are almost linear suggested that at these concentrations, apparent viscosity is independent of shear rate. The value of the flow behaviour index for yeast which has a suspension concentration of 10.5% wet weight was 0.71 (Table 5.1), although this is much lower than expected, it is associated with a rather high level of uncertainty (R^2 = 0.70). These results agree with those reported by Reuss *et al.* (1979) and Rudiš *et al.* (1976). The flow behaviour index, n decreases from 0.94 to 0.06 as the yeast suspension concentration is increased from 10.5 to 66.3% wet weight. Allen and Robinson (1990) reported similar results for filamentous broths although the flow behaviour index only decreased from 1 to values between 0.2 and 0.4. Allen and Robinson (1990) showed that for filamentous broths, there is a power law relationship between the consistency index and the biomass concentration with exponents that vary between 0.7 and 3.3. However, in this study the relationship between the consistency index and the suspension concentration was found to be represented by the exponential relationship (R^2 in the range 0.80 to 0.95) than the power law relationship (R^2 in the range 0.78 to 0.85).

In the concentration ranges above 20% wet weight, the rheology of the yeast suspension was shown to be non-Newtonian, more specifically approximated as pseudoplastic. The rheograms were fitted with different models: At yeast concentrations between 20 and 44.0% wet weight; the power law model fitted the data best whereas at higher yeast concentrations (58.0 to 66.3% wet weight), the Herschel-Bulkley model gave the best fit. Yeast suspensions at higher concentrations (58.0 to 66.3% wet weight) fitted the Herschel-Bulkley model because they had yield stresses that needed to be overcome before any fluid movement could take place. Aside from the evidence that the rheology of the yeast suspension at high concentrations could be approximated with the Herschel-Bulkley model from the shear stress-shear rate curves, it was physically observed that there was little fluid movement of highly concentrated yeast suspensions at low shear rates.

In Figure 5.3, the relationship between suspension concentrations and apparent viscosities is shown. The resulting curves were modelled with exponential, power law and linear fits as well as the Vand and Einstein equations. Reuss *et al.* (1979) obtained a hyperbolic relationship between volume fraction of *Saccharomyces cerevisiae* suspension, its packing factor (a function of osmotic pressure) and its relative viscosity (ratio of suspension viscosity and supernatant viscosity). This equation obtained by Reuss *et al.* (1979) was not considered in this study. Statistically, the power law, linear and Einstein equation fits were poor because of the low R^2 values obtained and so would be eliminated as suitable fits. Further, the Einstein equation is only applicable when the suspension concentration is less than 4% based on volume fraction (Reuss *et al.*, 1979). However, the Vand equation which is a good fit statistically ($R^2 > 0.97$) would be eliminated because it gave physically unfeasible apparent viscosity values between 15.1 and 28.5% wet weight. Thus, the relationship between the apparent viscosity and suspension concentration is exponential because the R^2 values of the regression lines were high (> 0.95) and the errors obtained (excluding the 20% wet weight) in using the equation to predict apparent viscosities fell within 23%. Rudiš *et al.* (1976) reported a cubic relationship between apparent viscosity and yeast suspension concentration. The reason for the difference is probably due to the range of yeast concentrations (between 0 and 10% wet weight) investigated by Rudiš *et al.* (1976). In conclusion, the nature of the rheology of the yeast suspension as well as magnitude of the apparent viscosity is a function of its concentration.

A relationship between the rheology of the yeast suspension and mixing time was established by investigating the effect of suspension concentration on mixing time. As the yeast concentration is increased from 30 to 40% wet weight, there is a small increase in the mixing time whereas an increase in concentration from 40 to 50% wet weight resulted in almost a 6-fold increase in mixing time as shown in Figures 5.6 and 5.7. Hence, the change in mixing time can be correlated largely with apparent yeast viscosity within these concentration ranges. The trend in mixing time shows an inverse relationship with the flow behaviour index, n (Table 5.1).

An investigation into the effect of agitation rate on mixing was conducted over a range of suspension concentrations. At suspension concentrations below 32% wet weight, mixing time is independent of agitation rate (Figures 5.4 and 5.5). Here, on increasing the agitation rate 4 fold, mixing times recorded were within 8% of the average, lying within the coefficient of variance of the analysis. However, as the cell concentration exceeded 37% wet weight, mixing time became increasingly dependent on agitation rate. At an agitation rate of 800 rpm, equivalent to a tip speed of 2.93 ms^{-1}, mixing time became independent of suspension concentration in the concentration range investigated. At low cell concentrations and high agitation rates, the apparent viscosity is low (Table 5.3) causing an increase in turbulence and an enhancement of homogeneity in mixing.

Of the two impeller types, the Rushton impeller gave better mixing than the pitched blade. At a yeast concentration of 30% wet weight, the mixing time obtained when using the Rushton impeller is low (< 20 s) and is relatively independent of the shear rate. This is because the

Rushton impeller dissipates more power than the pitched blade thereby leading to an increase in the energy available for mixing (Table 3.7). Further, the Rushton impeller gives radial mixing and has good viscosity tolerance which is useful when mixing highly viscous fluids (Rushton *et al*, 1950b).

In conclusion, to attain homogeneity within 5% of the time scale of the mixing process (i.e. homogeneity being attained in 12 minutes for a 4 hour mixing process), the Rushton impeller set at an impeller tip speed of 2.93 ms^{-1} is required for yeast concentrations ranging between 30 and 40 % wet weight. However, mixing time is only one criterion to consider on yeast storage. The maintenance of good quality yeast is imperative through minimisation of hydrodynamic stress and that implicated by nutrient deprivation. Further, since homogeneity was difficult to attain in the 55-65 % (based on wet weight) yeast suspension concentration range, it can be suggested that the yeast suspension is diluted to about 40 % wet weight (Figures 5.6, 5.7 and 5.8). In Figures 5.6 and 5.7, the order of magnitude of mixing time was the same in the range of yeast suspension concentrations between 30 and 45 % wet weight and on increasing the concentration to 53% wet weight; there was 10-fold increase in the mixing time. This observation confirms the need for dilution in stirred tank reactors. Effective mixing of diluted yeast (30 to 40 % wet weight) is possible at an impeller tip speed of 1.44 ms^{-1} (agitation rate of 400 rpm in the STR used for this study).

CHAPTER 6: EFFECT OF INTENSITY AND DURATION OF AGITATION ON YEAST QUALITY

6.1 INTRODUCTION

The effect of intensity and the duration of agitation on yeast quality are reported in this chapter. In order to explore the effect of the intensity of agitation, the impeller speed and the impeller type were changed. In Section 6.2, the investigation of the effect of impeller speed on yeast quality is reported while the effect of impeller type is treated in Section 6.3. Section 6.4 is concerned with the effect of agitation duration on yeast quality.

The methodology used to investigate the effect of intensity and duration of agitation on yeast quality is detailed in Chapter 3. The purpose of this set of experiments is to determine if there is deterioration in yeast quality on changing the intensity and duration of agitation and, if so, to establish a correlation between these.

6.2 EFFECT OF IMPELLER SPEED ON YEAST QUALITY

To investigate the effect of impeller speed on yeast quality, yeast suspension obtained from SABMiller Breweries in Newlands was agitated in a vessel of standard geometry at speeds of 200, 400, 600, 800 and 1200 rpm using a Rushton turbine of standard geometry. The duration of agitation was 8 hours and the temperature of jacketed vessel kept at 4°C. The concentration of the suspension was adjusted with PBS solution when necessary. Samples collected every two hours following agitation were analysed for viability, protease absorbance, vitality in subsequent small-scale fermentations and the amount of haze generated according to the methods detailed in Chapter 3.

6.2.1 VIABILITY

In Figure 6.1 and 6.2, the results obtained from investigating the effect of impeller speed on yeast viability are shown. For the control experiment, the yeast suspension at the concentration being investigated is stored in the fridge at 6°C without agitation. It is clearly shown in Figure 6.1 that on partial dilution of the cropped yeast to 42.0 and 56.2 % wet weight, there was no significant change in viability as impeller speed was increased. However, at an undiluted yeast concentration of 64.0% wet weight, a decline in viability of about 3.0% on agitation at 400 rpm and 8-hour storage is shown in Figure 6.2. The decrease in viability was slightly more at an agitation rate of 800 rpm. At 78.4% wet weight, there was a 3.0% decrease in viability at an impeller speed of 400 rpm and a 5% decrease in viability at an impeller speed of 800 rpm (Figure 6.2).

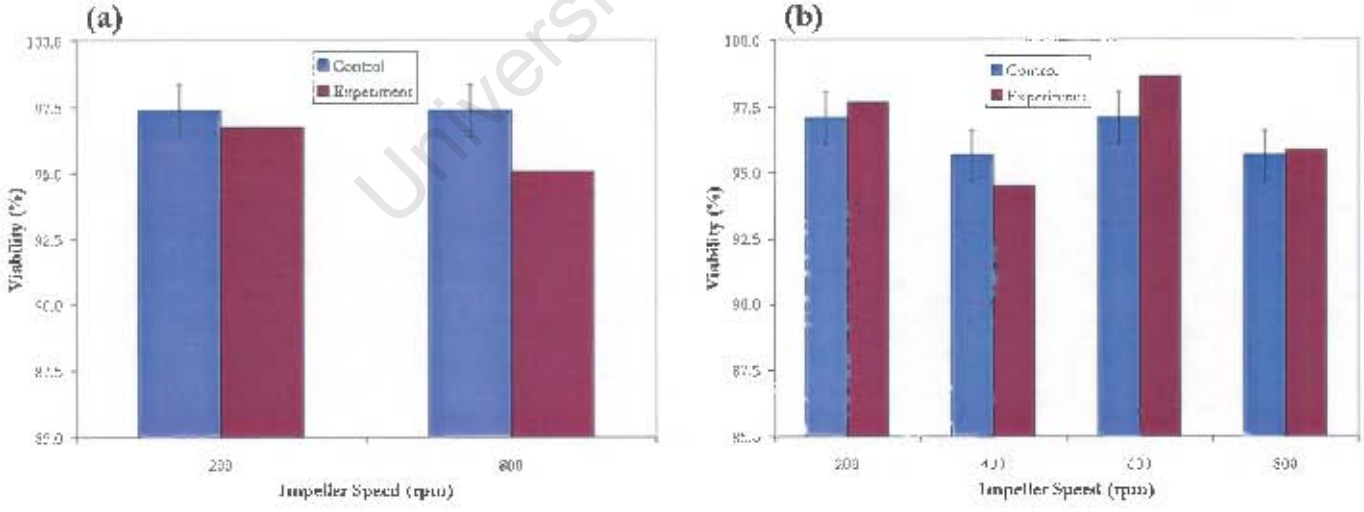

Figure 6.1: Effect of impeller speed on yeast viability for partially diluted yeast suspension following 8 hours agitation under a nitrogen blanket.
(a) - 42.0% wet weight, (b) - 56.2% wet weight

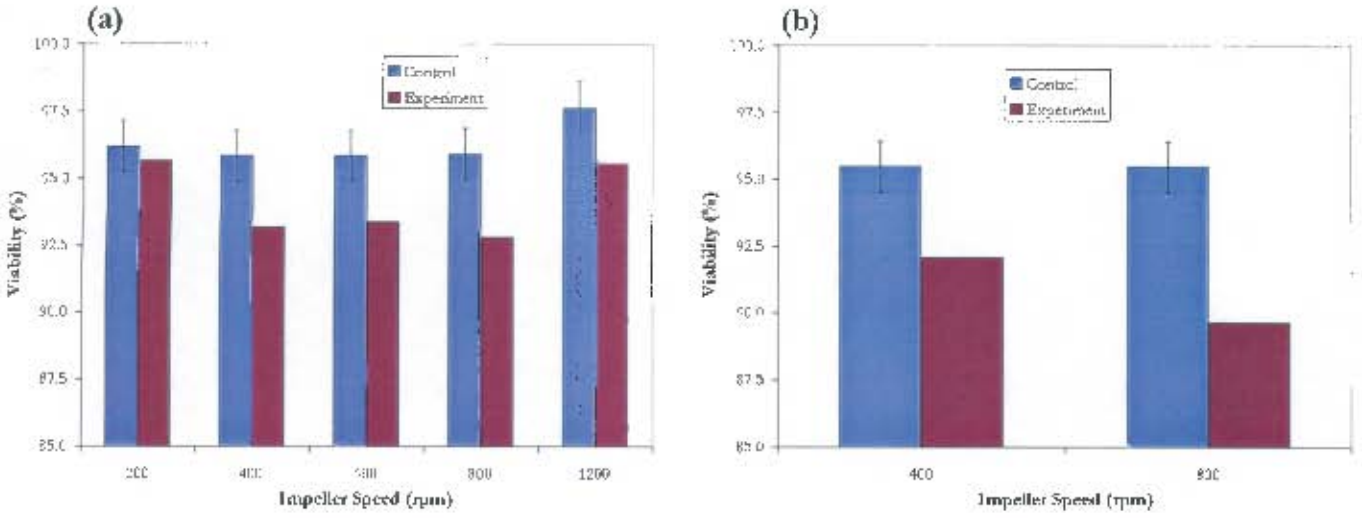

Figure 6.2: Effect of impeller speed on viability of undiluted yeast suspension following 8 hours agitation under a nitrogen blanket.
(a) - 64.0% wet weight and (b) - 78.4% wet weight

6.2.2 PROTEASE ABSORBANCE

The effect of impeller speed on protease release was investigated across the cell concentration range of 56.2 to 78.4% wet weight. Release of proteases from the yeast cell gives a measure of loss of membrane integrity. Results are presented in Figure 6.3. The high levels of protease detected in Figure 6.3a compared with Figures 6.3b – d results from the duration of incubation of 18 hours used at a yeast suspension concentration of 56.2% wet weight whereas the remaining incubation periods were 1 hour (Figures 6.3b, 6.3c and 6.3d). At a yeast suspension concentration of 56.2%, the increase in protease release was seen at an impeller speed of 800rpm. At 400 and 600 rpm, no significant variation in protease release between the control and experiment were observed. At a suspension concentration of 64.0% wet weight, the change in protease amount became more pronounced. At an impeller speed of 400rpm, the change in protease release was 0.005 while at 1200rpm the change increased to 0.027 (70% of control value). At an even higher suspension concentration (78.4% wet weight), there was a change in protease absorbance of 0.008 at an impeller speed of 400rpm and a change of 0.007 at an impeller speed of 800rpm. At all yeast concentrations above 60%, an increase in protease release was observed on agitation at 400 rpm or greater rates. At 56% yeast consistency, this increase was only observed at 800 rpm

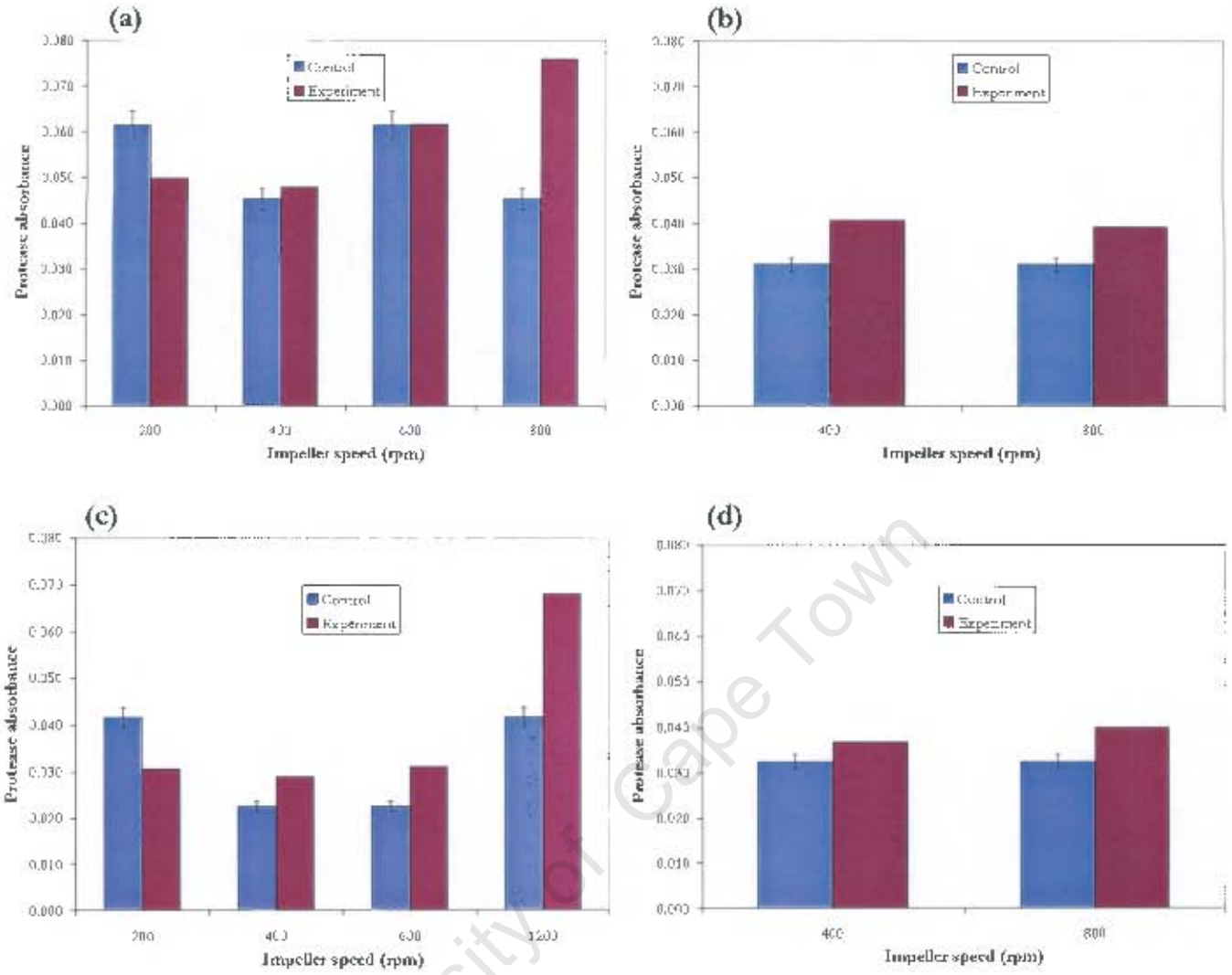

Figure 6.3: Effect of impeller speed on protease release (shown as absorbance) at varying cell concentrations. Control – yeast stored still and Experiment – agitated stored yeast.
(a) - 56.2% wet weight & 18 hour incubation (b) - 61.0% wet weight & 1 hour incubation, (c) - 64.0% wet weight & 1 hour incubation, (d) - 78.4% wet weight & 1 hour incubation

6.2.3 HAZE GENERATION

In Figure 6.4, the amount of haze generated following the agitation experiment is plotted as a function of the agitation rate. The amount of haze material in suspension was calculated as the ratio of the amount of haze obtained from the experiment to that obtained from the control where haze is measured in terms of particulate material less than 2 μm in diameter. The amount of haze increases as the impeller speed is increased to 600 rpm. At higher agitation rates, the amount of haze released decreases (Figure 6.4b).

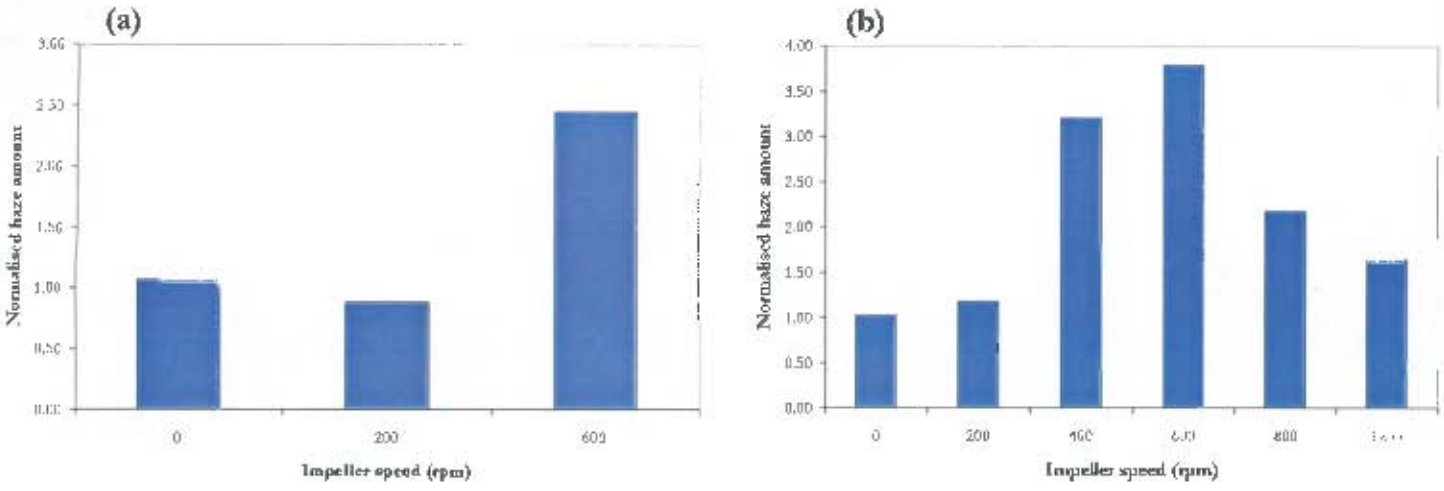

Figure 6.4: Effect of impeller speed on haze released following agitation with the Rushton turbine for 8 hours at 4°C

(a) – 55.4% wet weight yeast, (b) – 62.5% wet weight yeast

6.2.4 VITALITY

In order to assess yeast vitality, the experiment and control yeast was fermented in shake flasks anaerobically on MYPG media (Section 3.9.4) for 24 hours. The control yeast had been stored without agitation in the refrigerator at 6°C for 8 hrs. The raw data for these experiments are shown in Appendix D. The growth parameters obtained from the resultant growth curves of the control yeast and the yeast that had been subjected to agitation prior to being grown on MYPG media are shown in Tables 6.1 and 6.2. The general trend for the growth rate shows its decrease as the agitation rate of the prior storage conditions increased. The magnitude of this decrease exceeds the standard deviation of the measurement at an agitation rate of 600 and 800 rpm during storage. An increase in the biomass yield was observed on increasing the agitation rate to a maximum at 400 rpm whereafter this decreased by 2 to 4 fold. The change in the increase of biomass concentration (X_f/X_i) did not follow any particular trend while the exponential carbon dioxide yield followed the same trend as seen by the biomass yield i.e. a maximum carbon dioxide yield was found at 400 rpm. Finally, the substrate utilisation rate and carbon dioxide formation rate (exponential phase) also increased to a maximum at 400 rpm and thereafter decreased.

Table 6.1: Growth parameters for different impeller speeds

Speed (rpm)	μ (h^{-1})	$Y_{X/S}$ (10^9 cells/g)		X_f/X_i	$Y_{CO2/S}$ ($ngCO_2$/cell.g)	
		Exponential	Average		Exponential	Average
Control	0.047	0.32	0.08	1.64	3.04	0.28
200	0.068	0.52	0.11	2.07	3.65	0.37
400	0.054	0.71	0.11	1.21	5.02	0.51
600	0.018	0.27	0.04	1.29	4.05	0.28
800	0.021	0.18	0.07	1.55	3.09	0.30
Standard Deviation	±0.016	±0.10	±0.01	±0.16	±0.73	±0.06

Table 6.2: Utilisation and formation rates for different impeller speeds

Speed (rpm)	q_s (ng/h.cell)		q_{CO_2} (ng/h.cell)	
	Exponential	Average	Exponential	Average
Control	0.15	0.36	0.37	0.14
200	0.09	0.41	0.44	0.18
400	0.12	0.19	0.64	0.22
600	0.06	0.28	0.34	0.14
800	0.09	0.34	0.36	0.14
Standard Deviation	± 0.02	± 0.05	±0.25	±0.04

6.2.5 SUMMARY AND DISCUSSION OF IMPELLER SPEED EFFECTS

Yeast suspension that had been agitated at impeller speeds between 200 and 1200 rpm for 8 hours at 4°C was analysed for haze, protease, and viability and subjected to small-scale fermentation at 30°C for 24 hours. It is clear that as the impeller speed is increased, the viability of the cells and the amount of protease generated increased. Therefore, there is loss of cell membrane integrity as the agitation rate is increased and this is confirmed by the loss of viability. The amount of haze released into suspension reached a peak at 600 rpm while the impeller speed at which the metabolic activity of the cells is not compromised is optimum at 400 rpm. Thus, the subsequent performance of cropped yeast is dependent on prior storage agitation rate and the foam stability and beer flavour and taste is compromised at medium agitation rates.

The reason for the increase in the amount of haze as the impeller speed is increased is that as the agitation rate is increased, the yeast suspension becomes more turbulent and the mechanical abrasion of the cell wall is increased (Lewis and Poerwantaro, 1991) and the cell wall is one of the major constituents of haze. As the cell wall erodes, cell contents are released into the suspension and protease is found in the cell content which is the reason why the amount of protease increases with agitation rate. Erosion of the cell wall renders the cell wall porous which can lead to cell death and hence the reason for loss of viability.

6.3 EFFECT OF IMPELLER TYPE ON YEAST QUALITY

On investigating the effect of impeller type on yeast quality, two impellers were used: the Rushton turbine and 45° pitched blade impeller. These two impellers effect mixing by either radial mixing or axial mixing. The impellers were of standard geometry. The experiments were conducted at 4 and 14°C, across a range of concentrations and at agitation rates of 400 and 800 rpm. The control yeast was stored without agitation in a sealed bottle in the refrigerator at 6°C.

6.3.1 VIABILITY

Figure 6.5 relates how the different impellers affect yeast viability. From Figure 6.5, it is seen that the effect of impeller type is only noticed at a higher concentration of 60.7% wet weight. As presented in Section 6.2, at a suspension of 52.8% wet weight there is no significant change in viability even at an impeller speed of 800rpm. In comparison, there was 4.7% drop in viability in using the Rushton turbine as compared with a 1.2% drop in viability in using the pitched blade impeller at a cell concentration of 60.7% wet weight.

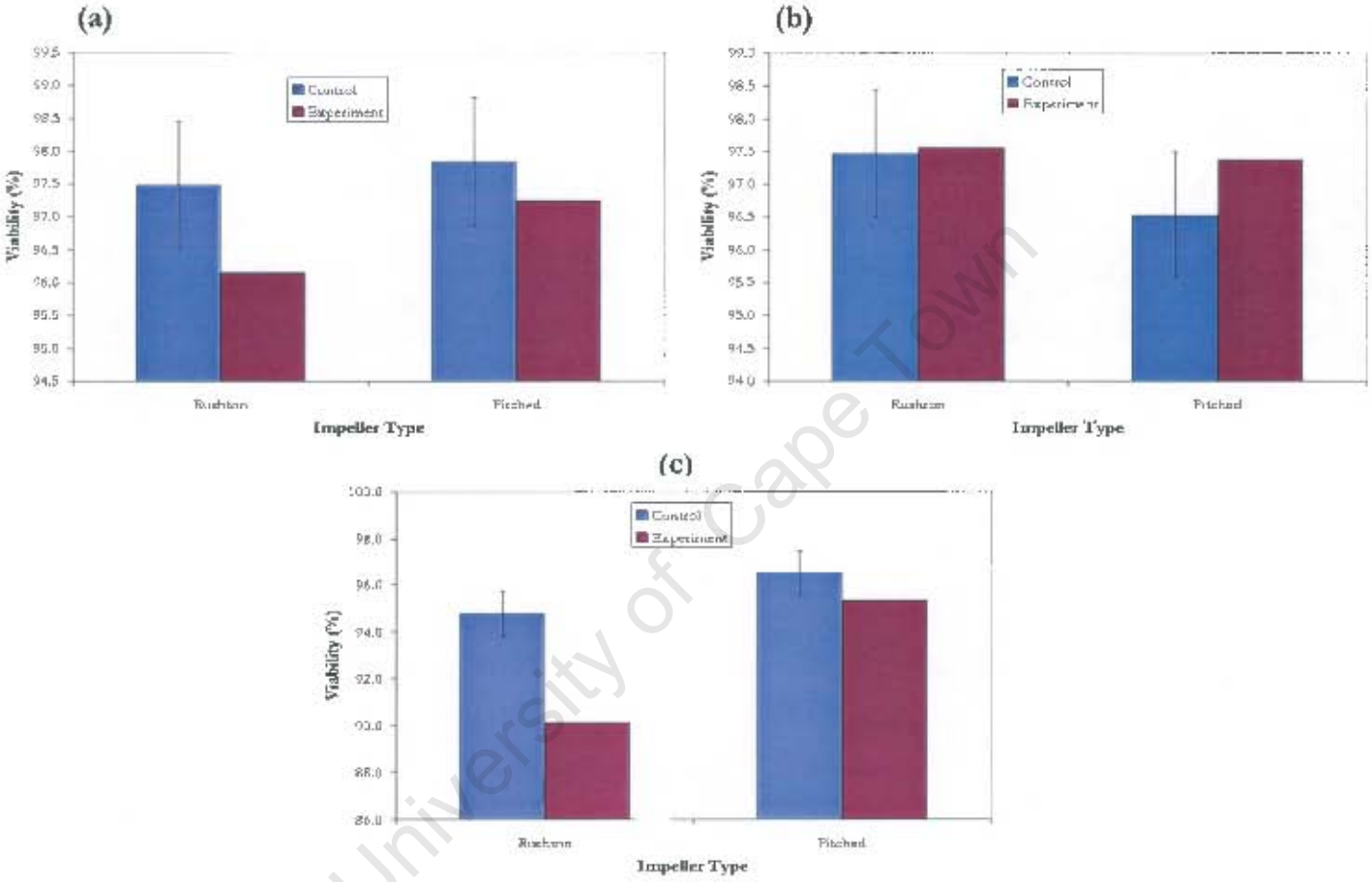

Figure 6.5: Effect of impeller type on viability
(a) - 52.8% wet weight, 4°C& 400 rpm (b) - 52.3% wet weight, 4°C & 800 rpm
(c) - 60.7% wet weight 14°C & 400 rpm

6.3.2 PROTEASE ABSORBANCE

The results of the relationship between protease absorbance and impeller type are shown in Figure 6.6. The yeast suspension used in this experiment had a concentration of 60.7% wet weight, the vessel temperature was at 4°C and the duration of agitation was 8 hours. In Figures 6.6, at the lower agitation rate of 400 rpm, there was an increase of 0.010 in protease absorbance using the Rushton turbine and an increase of 0.011 in protease absorbance with the pitched blade impeller. However, at an impeller speed of 800rpm the increase in protease absorbance was by 0.008 with the Rushton turbine and 0.002 with the pitched blade impeller.

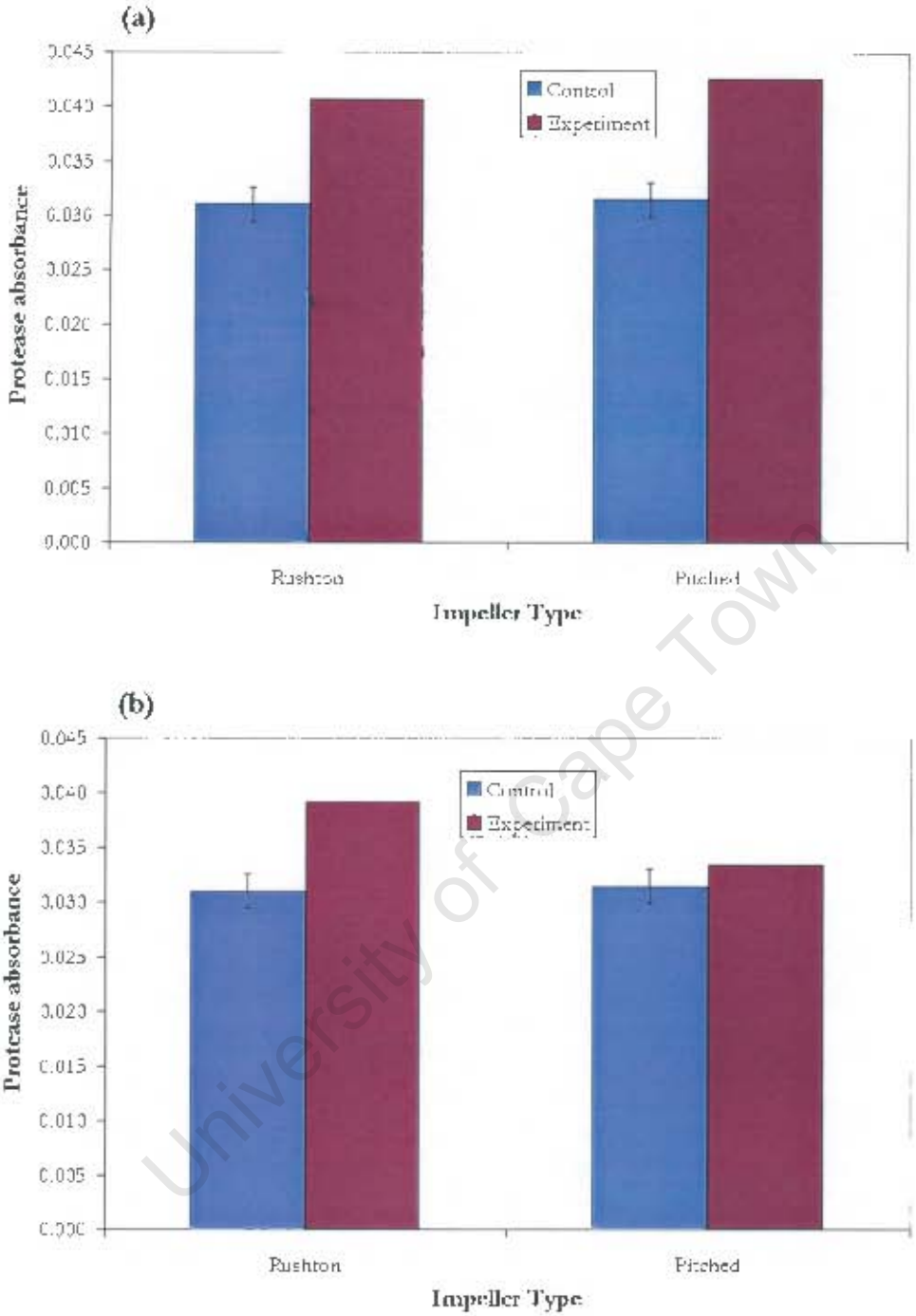

Figure 6.6: Change in protease absorbance as a result of different impeller types
(a) – 400 rpm and (b) – 800 rpm; (a) & (b) – Vessel temperature at 4 °C, Duration of agitation is 8 hours
Yeast concentration: 60.7% wet weight

6.3.3 HAZE GENERATION

The effect of impeller type on the amount of haze generated under agitation storage is shown in Figure 6.7. Change in the amount of haze is defined as the ratio of particulate material with diameter less than 2 μm in the yeast suspension whish had been stored for 8 hours and agitated compared with that which had been stored for 8 hours but not agitated (control). The

concentration of the yeast suspension used to obtain the data in Figure 6.7 is 60.3% wet weight. It can be seen that the Rushton turbine produced more haze than the pitched blade impeller. It seems that this effect is more pronounced at an agitation rate of 400 rpm. There was no additional haze generated on increasing the impeller speed from 400 to 800 rpm with the pitched blade impeller. However, on changing the impeller type from pitched blade to Rushton turbine there was a 3.2 fold increase on the amount of haze generated at 400 rpm. Further at an agitation rate of 800 rpm, the Rushton turbine generated 2.1 times more haze than the pitched blade impeller.

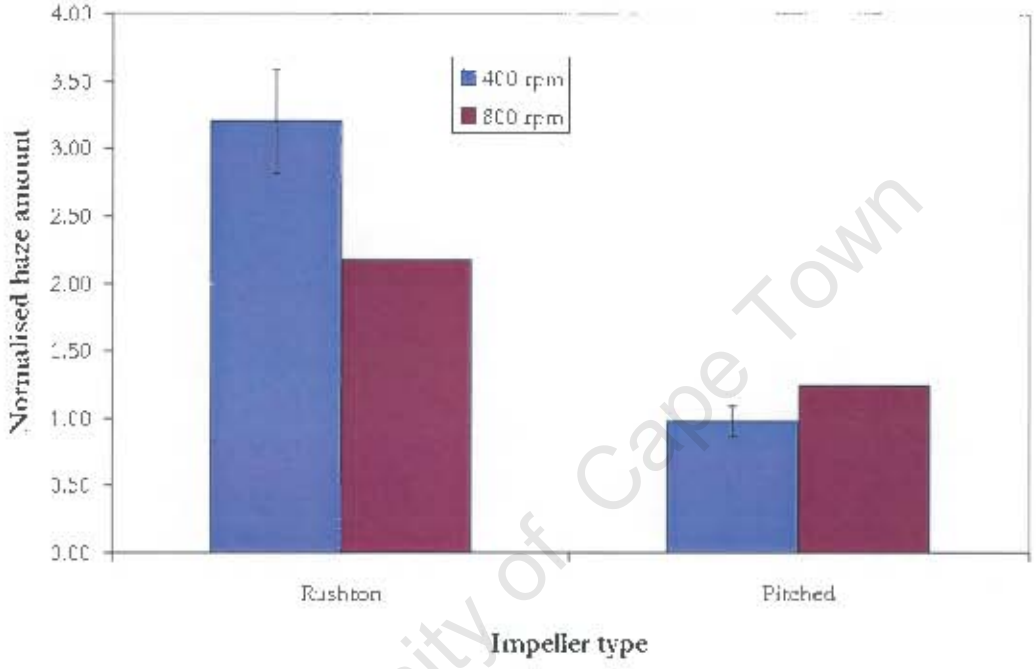

Figure 6.7: Effect of impeller type on haze amount on agitating for 8 hours at 4°C

6.3.4 VITALITY

In Table 6.3, the growth parameters obtained from the growth curves of yeast suspension that had been agitated by the two impellers at 4°C for 8 hrs before being grown under anaerobic conditions in MYPG media is shown. The growth rate in fermentation following storage under agitation by the Rushton turbine is less than that of the pitched blade impeller at 800 rpm by 0.05 h^{-1} and at 400 rpm, the difference between the growth rates is 0.01 h^{-1}. The difference in growth rates obtained from using the two types of impellers is significant at 800 rpm (standard deviation is 0.02 h^{-1}) but not significant at 400 rpm. The biomass yield data showed an increase in yield following agitation with the Rushton turbine when comparing pitched blade impeller with Rushton turbine at 400 and 800 rpm. In the case of the exponential phase carbon dioxide yield, there was a greater increase in the yield following agitation with the Rushton turbine compared to the pitched blade impeller. This can be interpreted as a greater requirement for metabolic energy under these conditions.

Comparing the two impellers at both speed settings, the yeast suspension agitated with the pitched blade impeller utilised glucose faster during the exponential phase than the Rushton turbine (Table 6.4). Finally, the specific rate of carbon dioxide formation is higher for Rushton turbine at 400 rpm, in agreement with the increase in the carbon dioxide yield.

Table 6.3: Growth Parameters for different impellers

Impeller Type	Speed (rpm)	μ (h^{-1})	$Y_{X/S}$ (10^9 cells/g)		X_f/X_i	$Y_{CO2/S}$ ($ngCO_2$/cell.g)	
			Exponential	Average		Exponential	Average
Pitched Blade	400	0.04	0.07	0.07	1.60	0.86	0.31
	800	0.07	0.13	0.05	1.47	0.87	0.36
Rushton Turbine	400	0.05	0.71	0.11	1.21	5.02	0.51
	800	0.02	0.18	0.07	1.55	3.09	0.30
Standard Deviation		±0.02	±0.02	±0.01	±0.18	±0.46	±0.04

Table 6.4: Substrate utilisation and CO_2 formation rates for different impellers

Impeller Type	Speed (rpm)	q_s (ng/h.cell)		q_{CO2} (ng/h.cell)	
		Exponential	Average	Exponential	Average
Pitched Blade	400	0.41	0.39	0.44	0.15
	800	0.43	0.37	0.47	0.18
Rushton Turbine	400	0.12	0.19	0.64	0.22
	800	0.09	0.34	0.36	0.14
Standard Deviation		±0.08	±0.06	±0.11	±0.07

6.3.5 SUMMARY AND DISCUSSION OF IMPELLER TYPE EFFECTS

Of the two impellers being investigated, the Rushton impeller effects more damage to the yeast cells than the pitched blade impeller. Both loss of viability (5% loss) and the loss of membrane integrity shown by viability and protease release are more pronounced when the Rushton turbine is used. The amount of haze found in suspension gives an indication of cell wall abrasion and integrity. The use of Rushton turbine causes a two-fold increase in the amount of haze found in suspension. Comparing the vitality of yeast suspension that had been agitated by the use of a Rushton turbine with that which had been agitated by a pitched blade impeller, it can be seen that the use of a Rushton turbine leads to a greater requirement of metabolic energy per unit growth as shown by comparison of the growth rates and yield on carbon dioxide. This is consistent with the greater cell damage predicted for the Rushton turbine because the radial flow pattern provides a higher shear environment. The increased flux of metabolic energy to withstand hydrodynamic stress, as proposed here, has been observed on growth of both *Saccharomyces cerevisiae* and *Sulfolobus* species in slurry bioreactors (Lamaignere, 2002; Sissing, 2001; Harrison *et al*, 2003).

6.4 EFFECT OF DURATION OF AGITATION ON YEAST QUALITY

Experiments were conducted to compare the effect of the duration of agitation over 4 hrs, 8 hrs and 24 hrs on yeast quality. For this set of experiments, the Rushton turbine was used at impeller speeds of 400 and 600 rpm (1.47 and 2.20 ms^{-1}). The temperature was kept constant at 4°C. As the control, a yeast suspension that was stored in the refrigerator at 6 °C without agitation for the duration of the experiment.

6.4.1 VIABILITY

The effect of the duration of agitation on yeast viability following agitation by a Rushton turbine at 400 rpm and 600 rpm at a temperature of 4°C is presented in Figure 6.8. The concentrations of the yeast suspension used were 51.8 and 62.2 % wet weight. The control yeast suspension was stored in the fridge for a duration of 24 hrs at 6°C without agitation. The vertical axes in Figure 6.8 show the decrease in viability calculated as the difference between the viability of the control yeast suspension and that of the experiment. At a yeast concentration of 51.8% wet weight and an impeller speed of 400 rpm, the viability did not change significantly with the duration of agitation. By the 24th hour the loss in viability amounted to 0.6% which is not a statistically significant change as the standard deviation for the viability assay is 0.95%. At an impeller speed of 600 rpm and yeast concentration of 51.8% wet weight, no consistent trend in viability was seen with time. The increase that occurred in the 24th hour may be attributed to oxygen entrainment during sampling. At a yeast concentration of 62.2% wet weight and an impeller speed of 400 rpm, the change in viability is only significant at the 24th hour of agitation (a decrease of 1.9%). However, at a concentration of 62.2% wet weight and 600 rpm, a decrease in viability in the range 1.1 to 0.9% from 4 to 24 hours of agitation is observed. However, the maximum decrease occurred in the 8th hour.

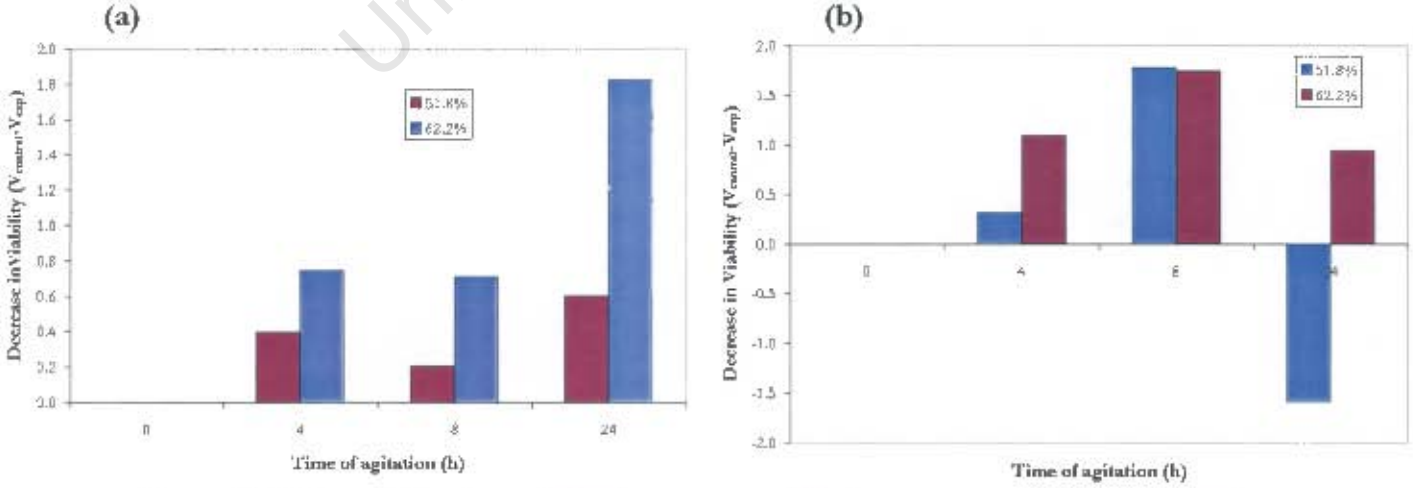

Figure 6.8: Effect of duration of agitation on yeast viability on storage at 4°C using Rushton turbine. (a) – 400 rpm, (b) – 600 rpm

6.4.2 PROTEASE ABSORBANCE

Release of intracellular protease under the same conditions discussed in Section 6.4.1 is presented in Figure 6.9. According to the statistical analysis done on the protease absorbance in Section 4.3.2, protease release is only significant (95% confidence limit) if the change in absorbance is more than 0.002. At a yeast concentration of 51.8% wet weight and an impeller speed of 400 rpm, there is an increase of 0.014 absorbance units in the amount of protease released in the 1st 4 hours, an increase of 0.001 in the 8th hour and a decrease of 0.003 in the 24th hour. At a concentration of 62.2% wet weight, there was an increase in the amount of protease release throughout. At the 4th hour, the change in protease absorbance amounted to 0.013, at the 8th hour, it almost doubled to 0.025 while at 24 hours, the change was found to be 0.040. The control yeast also experienced an increase in protease absorbance of 0.010. Finally, at 600 rpm and a suspension concentration of 51.8% wet weight, there is a slight increase of 0.007 in the amount of protease released in the 4th hour, a slight decrease of 0.006 in the 8th hour and a change of 0.008 in the 24th hour. However at a higher concentration of 62.2% wet weight, the increase in the 4th hour is almost a 100%, this decreases to an increase of 67% in the 8th hour and finally to an increase of 29.0% in the 24th hour. For the yeast suspension with a concentration of 51.8%, the change in protease absorbance is most significant in the 4th hour of agitation while in the case of the yeast suspension with a concentration of 62.2%, the most significant change in protease is in the 4th hour at an agitation rate of 600 rpm and in the 24th hour at an agitation rate of 400 rpm. The control yeast also experienced an increase in the amount of protease release of 39.4% (0.012 in protease absorbance) over the 24-hour period.

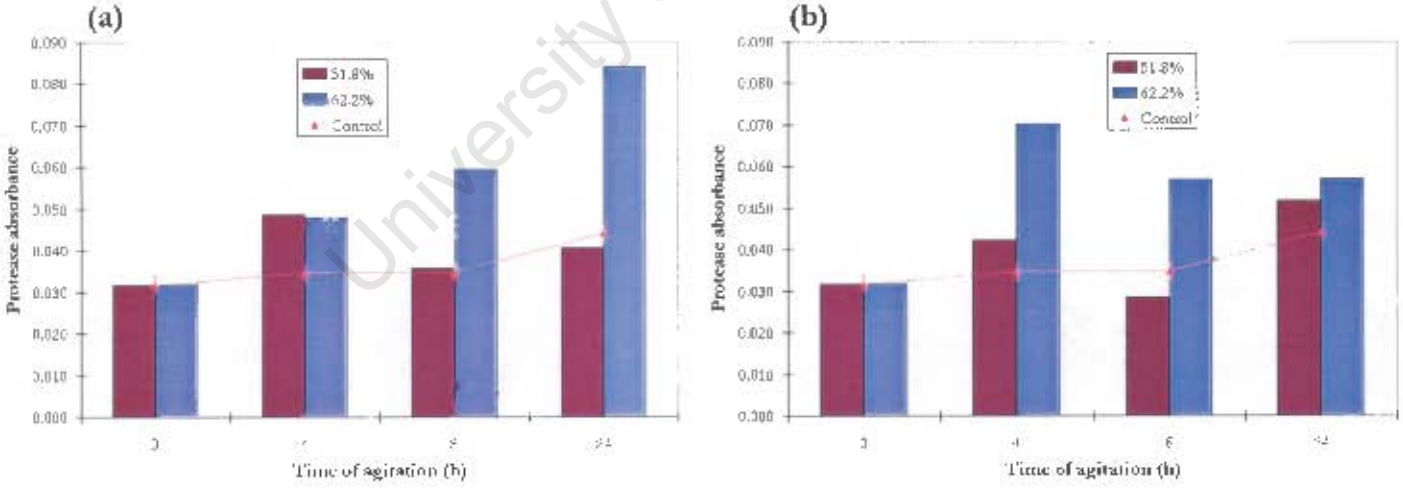

Figure 6.9: Effect of duration of agitation on protease absorbance using Rushton turbine
(a) – 400 rpm, (b) – 600 rpm

6.4.3 HAZE GENERATION

The generation of haze as a function of the duration of agitation was studied using a Rushton turbine at 600rpm, a yeast concentration of 61.0% wet weight, and a temperature of 4°C. Data is presented in Figure 6.10. As the duration of agitation increases the amount of haze found in the suspension also increased. In Figure 6.11, the effect of duration of agitation on the amount of haze released is shown using yeast suspension with a concentration of 62.2% wet weight at impeller speeds of 400 and 600 rpm. The amount of haze increases as the duration of agitation increases and the effect is more pronounced at 600 rpm.

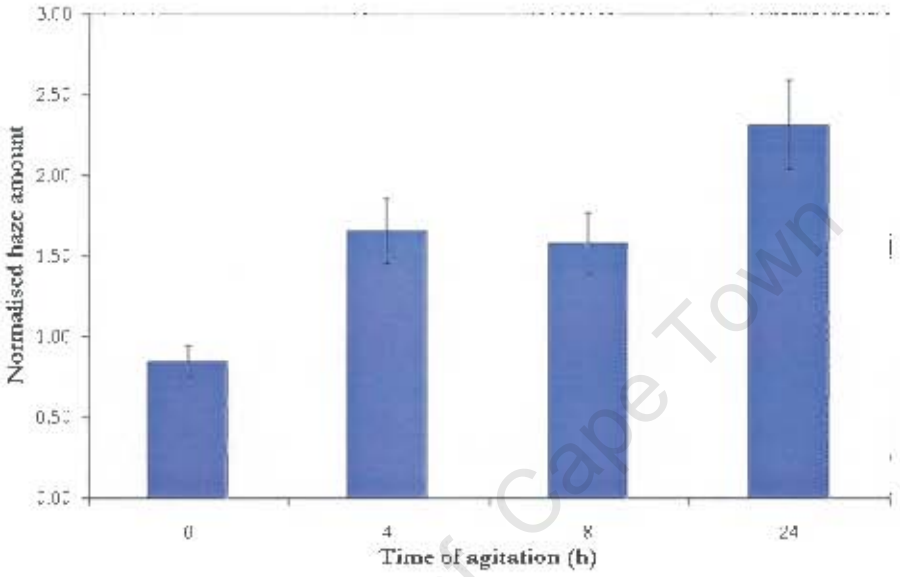

Figure 6.10: Effect of the duration of agitation on the amount of haze released using Rushton turbine at 600 rpm and 4°C. Yeast suspension concentration is 61.0% wet weight

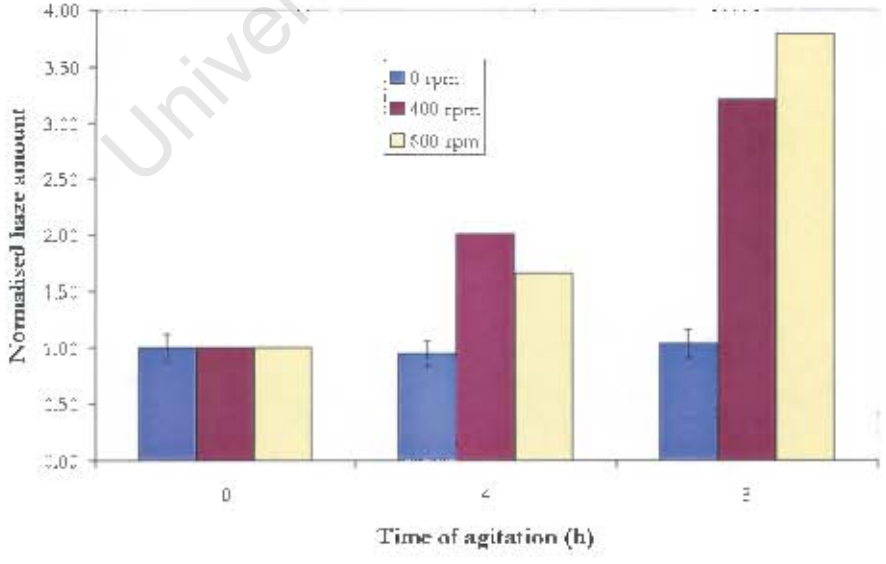

Figure 6.11: Effect of duration of agitation on the amount of haze released using Rushton turbine at 4°C. Yeast suspension concentration is 62.2% wet weight.

6.4.4 VITALITY

To investigate the effect of the duration of agitation on yeast quality, a yeast suspension that had been agitated for 4 hours and 8 hours at 4°C before being grown in MYPG media for 24 hours were compared. The yeast suspension of 56.0% wet weight was agitated by the Rushton turbine at rates of 0 to 800 rpm and a temperature of 4°C prior to fermentation. In Figure 6.12, 4-hour data are compared with 8-hour data across a range of impeller speeds. The growth rates are less for the suspension that was agitated for 8 hours as compared with the suspension that was agitated for 4 hours except for the 200 rpm data. The exponential phase biomass yield is significantly more for the suspension agitated for 8 hours but as the speed increases above 400 rpm, the difference reduces. In addition, the exponential phase carbon dioxide yield shows a difference when comparing across durations however, the change in biomass concentrations does not follow any particular trend.

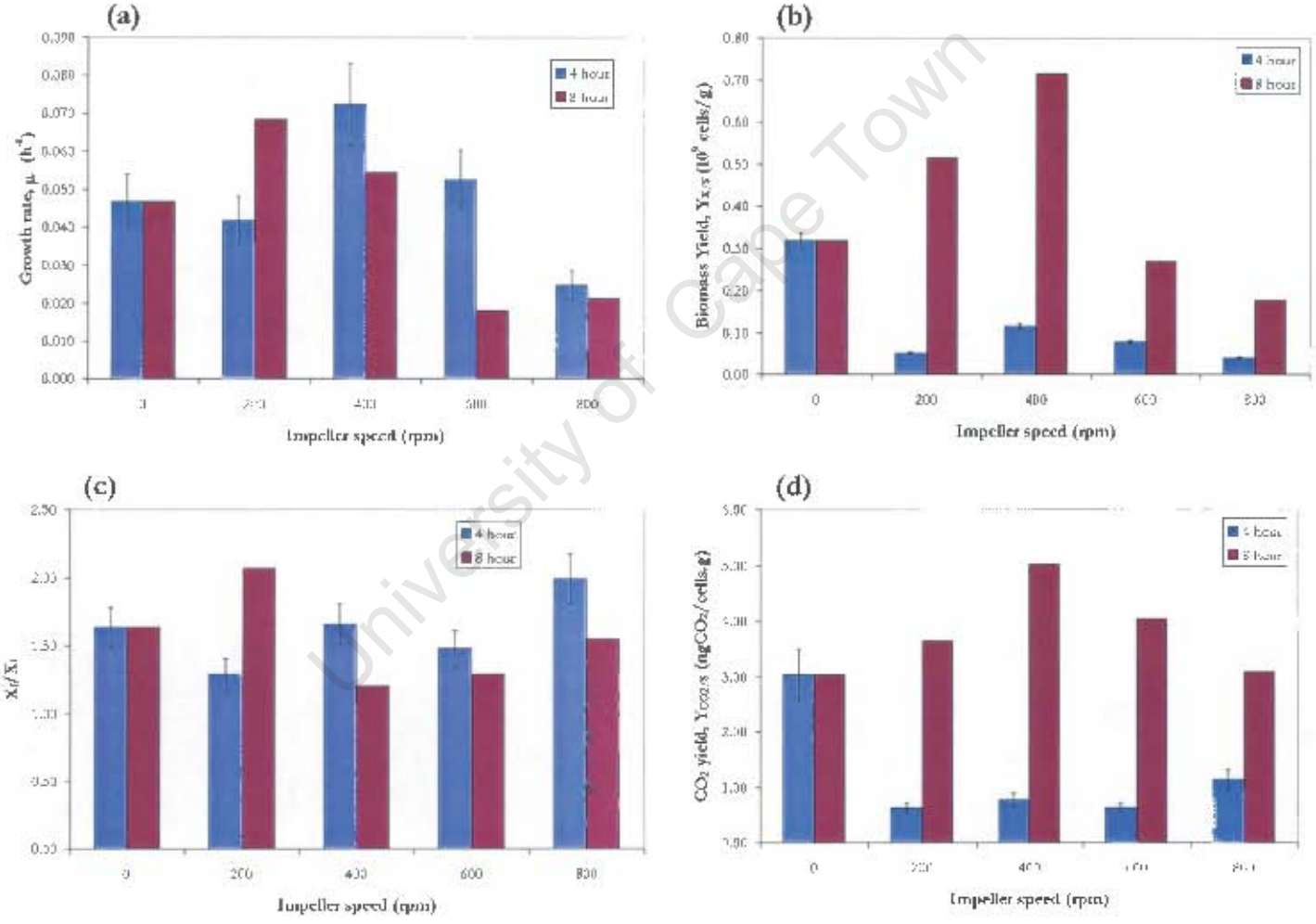

Figure 6.12: Effect of duration of agitation on growth parameters
(a) – Growth rate; (b) – Biomass yield; (c) – Increase in biomass concentration; (d) – Carbon dioxide yield

Comparing the exponential phase glucose utilisation rates (see Figure 6.13) it can be stated that the suspension that had been agitated for 4 hours prior to fermentation utilised glucose at a faster rate than that which had been agitated for 8 hours. Nonetheless, the carbon dioxide

formation rates are slightly different for the two durations of agitation except at 600 rpm, where the difference is more pronounced.

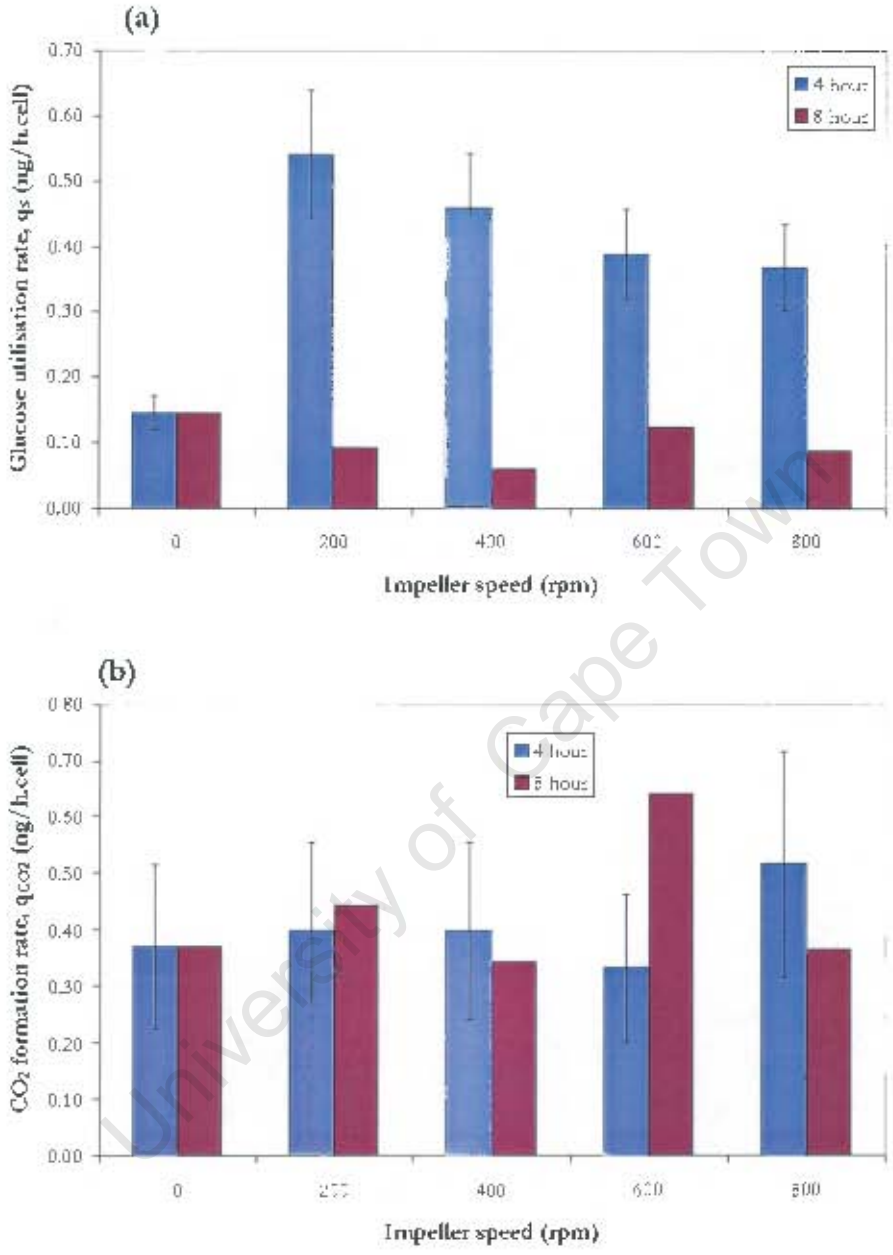

Figure 6.13: Effect of duration of agitation on glucose utilisation and CO_2 formation rate
(a) – Glucose utilisation rate, (b) – Carbon dioxide formation rate

6.4.5 SUMMARY AND DISCUSSION OF EXPOSURE TIME EFFECTS

In investigating the effect of duration of agitation on yeast quality, yeast suspension was agitated for 24 hours at 400 rpm and 600 rpm and then assayed for yeast quality indicators. The more concentrated suspension (62.2% wet weight) experienced a more pronounced decrease in viability and an increase in protease release at both speed settings and these increased with increasing duration of exposure indicating that the long-term exposure to agitation leads to a loss of viability and membrane integrity. However, on dilution of the yeast

suspension to 51.8%, the negative effect of long-term agitation on viability and protease release is less pronounced and not statistically significant. The effect of long-term agitation on the amount of haze released echoes that of protease release in that the amount of haze increased as the period of exposure increased over 24 hours. Finally, in looking at vitality, long-term agitation renders the yeast cells to require more metabolic energy. In other words, the cells that have been agitated for a longer period do not perform as well in subsequent fermentations.

Agitating the yeast suspension for a long period exposes them to two possible factors by which the yeast quality could deteriorate. One is through long-term storage effects and the other is by long-term exposure to a high-shear environment. The long-term exposure to agitation seems to be the more significant factor, as the control yeast did not experience as much loss in yeast quality as the agitated yeast.

6.5 Chapter Discussion And Conclusions

In this chapter the effect of the intensity and duration of agitation on yeast quality was investigated. Intensity of agitation is a function of the geometry, type and the speed of the impeller used. Impeller geometry was eliminated from the factors being investigated by using impellers of standard geometry. A range of impeller speeds from 200 to 1200 rpm and two types of impellers (Rushton turbine and 45° pitched blade) were used to alter the intensity of agitation. It was concluded that the more intense the agitation, the more pronounced the loss of viability and loss of membrane integrity. These results are supported by McCaig and Bendiak (1985a) who observed a reduction in the viability of pitching yeast agitated at a rate of 0.72 ms^{-1} at 1°C for 5 days. Subsequent fermentations become less successful if the impeller speed was increased above 400 rpm and if Rushton turbine was used at the higher speed settings (> 400 rpm). The amount of haze released into suspension has a peak at 600 rpm. The result obtained in this study is different from that obtained by Lewis and Poerwantaro (1991) who concluded that the duration of agitation was the only factor that contributed to an increase in the amount of haze released. The amount of haze released in their study was independent of the intensity of agitation and the temperature. It must be noted however, that the agitation rates used in their study were quite low (200 and 300 rpm).

An investigation into the effect of the duration of agitation on yeast quality was conducted by agitating the yeast suspension for a period of 24 hours and sampling at 4 hours, 8 hours and 24 hours and then analysing for yeast quality indicators. It was observed that the longer the period of agitation, the more pronounced the loss of yeast quality and this is highly dependent on the concentration of the yeast suspension. These results are similar to those obtained by Lewis and Poerwantaro (1991). McCaig and Bendiak (1985b) also stated that although the long duration of yeast storage affects its viability, it does not affect subsequent fermentations.

These results are in contrast with those obtained in this study, in that the growth rates of yeast which had been stressed by intense agitation and subjected to long durations of agitation were less than the growth rates of yeast that had not been agitated or agitated for a shorter period. In conclusion, to avoid deterioration of yeast quality, it is best to operate a 1.00l storage vessel of standard geometry at medium impeller speed (300 - 400 rpm) and the exposure time to agitation should not be longer than 8 hours.

CHAPTER 7: EFFECT OF SUSPENSION CONCENTRATION ON YEAST QUALITY

7.1 INTRODUCTION

In this chapter, the results obtained experimentally to ascertain the effect of changing suspension concentrations on the indicators of yeast quality are shown. The comprehensive methodology by which the data reported in this chapter was collected is detailed in Chapter 3. The aim of these experiments is to evaluate how the changes in suspension concentration affect the yeast quality indicators. Section 7.2 reports the effect on viability, Section 7.3 the protease absorbance, Section 7.4 the haze generation and lastly, Section 7.5 the effect on vitality.

An aliquot of 0.91 l of yeast suspension at concentrations ranging from 40 to 60% wet weight were placed in the experimental rig and agitated at impellers speeds ranging from 200 to 800 rpm. The impeller used for this investigation was the Rushton turbine and temperature of the vessel was held at 4°C unless otherwise stated. Samples were taken every 2 hours and analyzed for viability by the use of methylene blue staining method, protease absorbance by the method proposed by Robinson (2001), haze generated and small-scale fermentation performance.

7.2 VIABILITY

In Figure 7.1, the yeast viability measured by methylene blue staining is reported at different suspension concentrations. The relationship between suspension concentration and viability has been compared across impeller speeds (200, 400 and 800rpm) and temperatures (4 and 14°C). At 200rpm, there is no significant change in viability over the range of concentrations shown (41.0 - 65.6% wet weight). However as the agitation rate increases to 400rpm (impeller tip speed of 0.72 ms^{-1}), a loss of viability is only noticeable at higher suspension concentrations such as a decline of 2.5% at 63.0% wet weight at 4°C and a decline of 3.4% at 78.4% wet weight at 14°C. At an impeller speed of 800 rpm, the loss of viability is even more pronounced; a decline of 5.8% is seen in Figure 7.1d at an agitation rate of 800 rpm and 14°C. Loss of yeast quality as shown by viability is more pronounced the more concentrated the yeast suspension is.

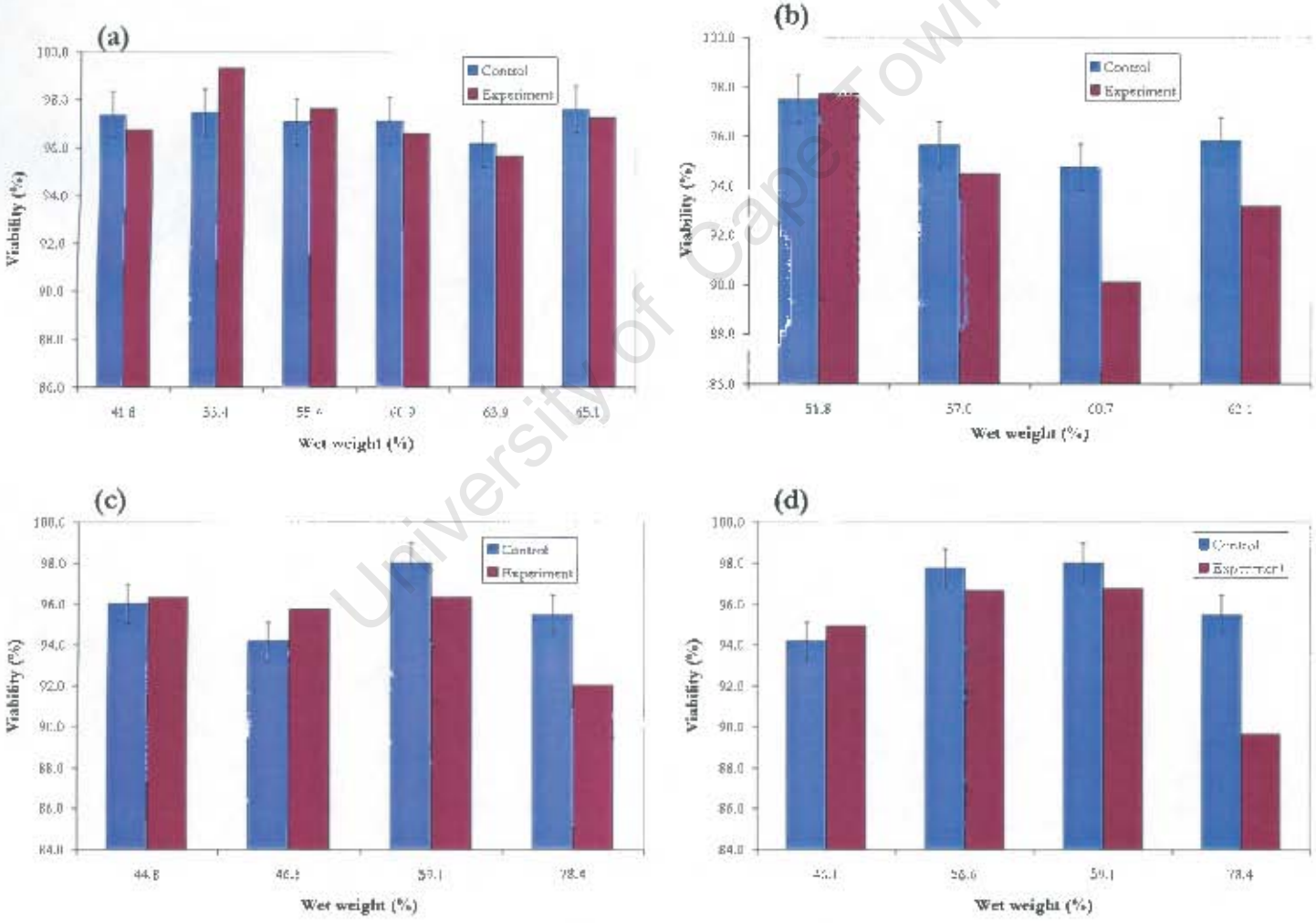

Figure 7.1: Change in viability with cell concentration on agitation with the Rushton turbine for 8 hours.

(a) - 200rpm & 4°C, (b) - 400rpm & 4°C, (c) - 400rpm & 14°C, (d) - 800rpm & 14°C

7.3 PROTEASE ABSORBANCE

Protease release was measured by using the method proposed by Robinson (2001). In Figure 7.2, the general trend across concentrations is that protease release increases as concentration increases. There is a substantial amount of protease release in the lower concentration ranges at 200 rpm and 4 °C (0.016 at 41.8% wet weight and 0.018 at 55.4% wet weight). However at 400 rpm and 4 °C, the amount of protease release increases, 56% increase at 51.8% wet weight, 68% increase at 57.0%, 96% increase at 61.2% wet weight and an increase of 123% at 63.1% wet weight. Finally at 14 °C, the increase in protease release across concentrations is not as pronounced. The results illustrate that there is loss of membrane integrity on agitation under refrigerated conditions as suspension concentration increases. This suggests that yeast quality on storage is influenced by the temperature history of the yeast, with resilience decreased by exposure to decreasing temperature. This is further discussed in Chapter 8.

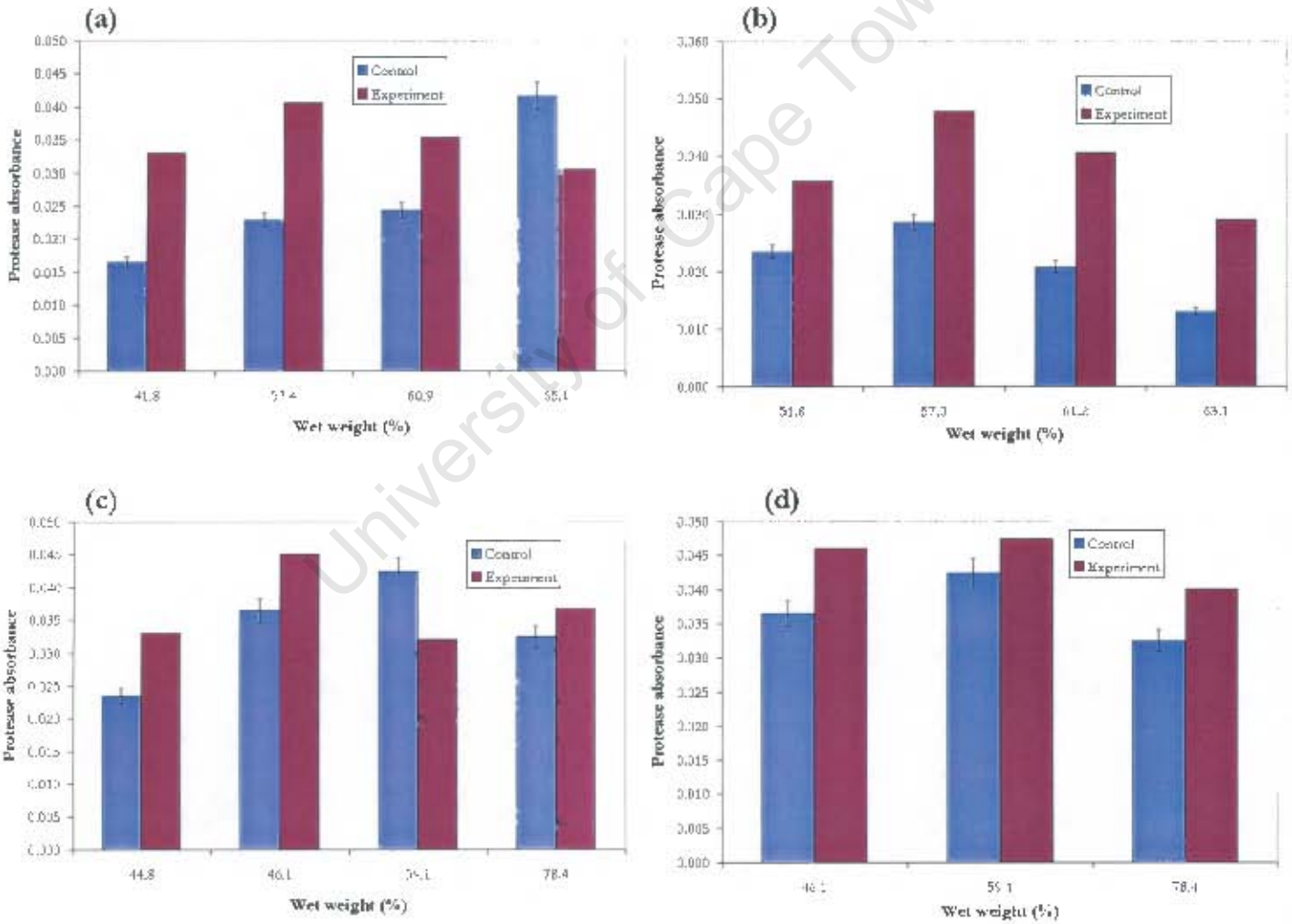

Figure 7.2: Change in Protease absorbance with suspension concentration on agitation with the Rushton turbine for 8 hours.

(a) - 200rpm & 4°C, (b) - 400rpm & 4°C, (c) - 400rpm & 14°C, (d) - 800rpm & 14°C

7.4 HAZE GENERATION

The amount of haze found in suspension can affect beer clarity (O'Connor-Cox, 1994) and downstream processing (Siebert *et al.*, 1987) hence, the amount of haze found in suspension is one of the indicators of yeast quality. In Figure 7.3, the relationship between the increase in the amount of haze (Normalised with respect to control) and suspension concentration is shown. This data was obtained by agitating the yeast suspension with varying concentrations at 200 rpm and 600 rpm and 4°C and then analysing for the amount of haze (sub– 2µm) by the use of the Malvern mastersizer (See Section 3.9.4) The increase in the amount of haze has been defined as the ratio between the amount of haze found in treated yeast and that found in the control yeast. The normalised haze amount increases only slightly as the suspension concentration increases at an agitation rate of 200 rpm. However, at an agitation rate of 600 rpm, the amount of haze generated and the increase in haze as the concentration increases is more pronounced. This result suggests that the amount of haze is dependent on the concentration of the suspension. Consequently, the more concentrated the yeast suspension is, the higher the probability that the yeast quality is compromised.

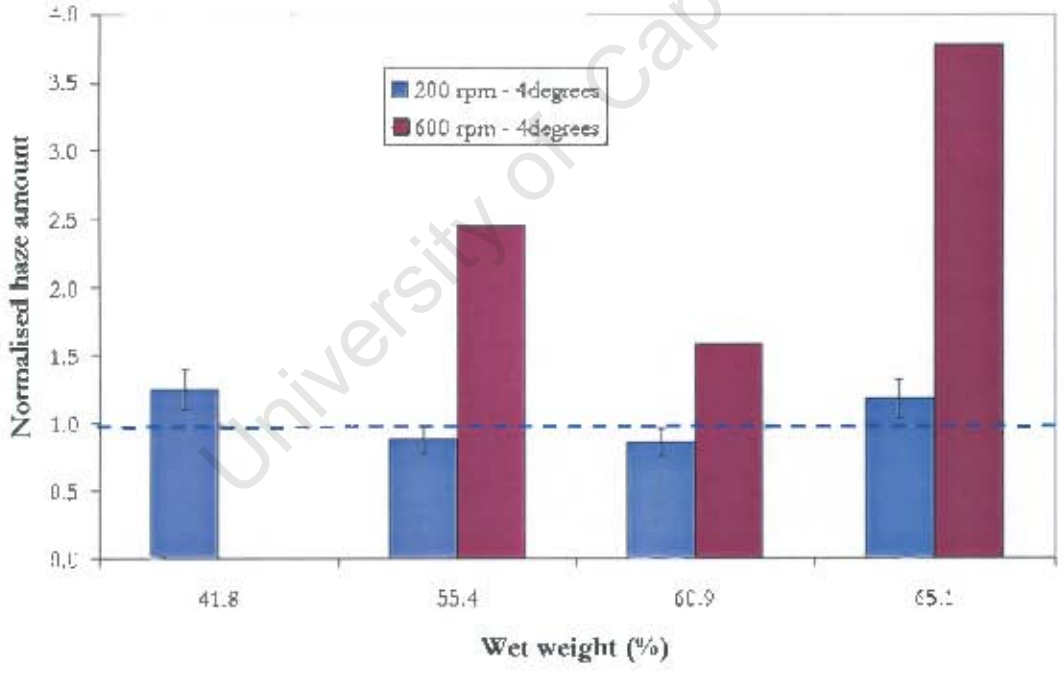

Figure 7.3: Effect of yeast suspension concentration on the amount of haze released

Legend: - - - - - - - - Normal line

7.5 VITALITY

The sets of data shown in Table 7.1 and Table 7.2 were obtained by agitating the yeast suspension with Rushton turbine at an agitation rate of 800 rpm and a temperature to 14°C. In Table 7.1, the growth parameters: growth rate, yields and change in cell concentration from the beginning to the end of the fermentation process have been tabulated. In Table 7.2, the substrate utilization rates and glucose formation rates are shown. The growth rate decreasesd by 77.8%, the yield decreased by 66.7%, the change in cell concentration decreased by over a 100%. The change in the carbon dioxide yield was negligible as the suspension concentration increases from 46 to 78% wet weight (Table 7.1). From Table 7.2, it can be seen that the substrate utilization rate decreased by 100% while the carbon dioxide formation rate was not affected by the increase in suspension concentration. Hence, the performance of yeast suspensions in subsequent fermentations is expected to deteriorate as the suspension concentration increases in the range 45 to 78% wet weight.

Table 7.1: Growth Parameters during fermentation following agitation under storage at different cell concentrations

Wet weight (%)	μ (h^{-1})	$Y_{X/S}$ (10^9 cells/g)	X_f/X_i	$Y_{CO2/S}$ ($ngCO_2$/cells.g)
46.1	0.09	0.09	2.49	0.35
78.4	0.02	0.03	1.23	0.30
Standard Deviation	±0.02	±0.01	±0.22	±0.04

Table 7.2: Substrate utilization and CO_2 formation rates during fermentation following agitation under storage at different concentrations

Wet weight (%)	qs (ng/h.cells)	q_{CO2} (ng/h.cells)
46.1	0.68	0.17
78.4	0.33	0.16
Standard Deviation	±0.08	±0.02

7.6 Discussion And Conclusions

In this chapter the effect of dilution of the yeast suspension during storage on yeast quality is tested. Cropped yeast was diluted into PBS to get yeast suspension of varying concentrations and the resultant suspensions were then subjected to mechanical stress by agitating with Rushton turbine at impeller speeds varying between 200 and 800 rpm and a vessel temperature of 4 and 14°C. After the yeast suspension had been subjected to mechanical stress, it was then assayed for protease release, viability and haze present. It was also used as inoculum for anaerobic fermentation in a shake flask for 24 hours. It is clearly seen from the results obtained that the yeast quality deteriorated as the suspension concentration of the storage phase increased. The loss of yeast quality can be attributed to an increase in the number of cells present in an aliquot of suspension; which increased the rate of agitation of inter-particle interactions. Another reason for deterioration in yeast quality could be because the efficiency of mixing was greatly reduced as the yeast concentration increased (Section 5.9). An increase in the cell concentration leads to a power increase in the mixing time of the order of 3 and this increase in mixing time shows the magnitude by which the efficiency of mixing is reduced. The yeast suspension used in this study was not centrifuged to remove all traces of ethanol and then re-suspended in PBS before being subjected to agitation. The presence of ethanol in the yeast suspension can induce heat shock proteins and enhance cell ageing (Walker, 1998). On diluting the yeast suspension, the concentration of ethanol is reduced and hence, a reduction in possible ethanol stress.

CHAPTER 8: EFFECT OF TEMPERATURE ON YEAST QUALITY

8.1 INTRODUCTION

In this chapter the results obtained from investigating the effect of temperature on yeast quality are reported. The method by which the data used in this chapter were collected is reported in Chapter 3 and the reproducibility of the data shown in Chapter 4. The aim of these experiments is to determine how the changes in the storage temperature in a mixing vessel affect the yeast quality of stationary phase yeast. In Section 8.2, the effect of temperature on viability is discussed. Section 8.3 reports the effect of storage temperature on the amount of protease released, Section 8.4 on the amount of haze generated and Section 8.5 on the effect on vitality.

The yeast suspension is kept in a temperature-controlled environment in the YCV. Before the yeast suspension gets to the YCV in the brewery process, it would have been cooled down from 14 to 4°C in a heat exchanger. Due to this, this study investigates the effect of temperature on yeast quality. The storage temperatures investigated in this study were 4 and 14°C. Rushton turbine at impeller speeds of 400 and 800rpm was used and the suspensions used ranged between concentrations of 44.0% and 60.7% wet weight. Samples were taken every 2 hours and analysed for yeast viability, vitality, protease released and the amount of haze generated.

8.2 VIABILITY

Yeast viability was measured by the use of the methylene blue staining method. The difference between the viabilities of two yeast samples is said to be significant if it is greater than 1.1%. In Figure 8.1, the relationship between the storage temperature and yeast viability is shown. In Figure 8.1a, the effect of storage temperature on yeast suspension with a concentration of 48.9% wet weight is shown and Figure 8.1b is for yeast suspension with a concentration of 59.9%. In both cases, Rushton turbine at an agitation rate of 400 rpm (impeller tip speed of 0.72 ms^{-1}) was used. At 48.9% wet weight, there is no significant change in viability across the two temperature settings. However, at a higher suspension concentration of 59.9%, there is no statistically significant reduction in viability at 14°C whereas there is a 3.2% reduction in viability at 4°C.

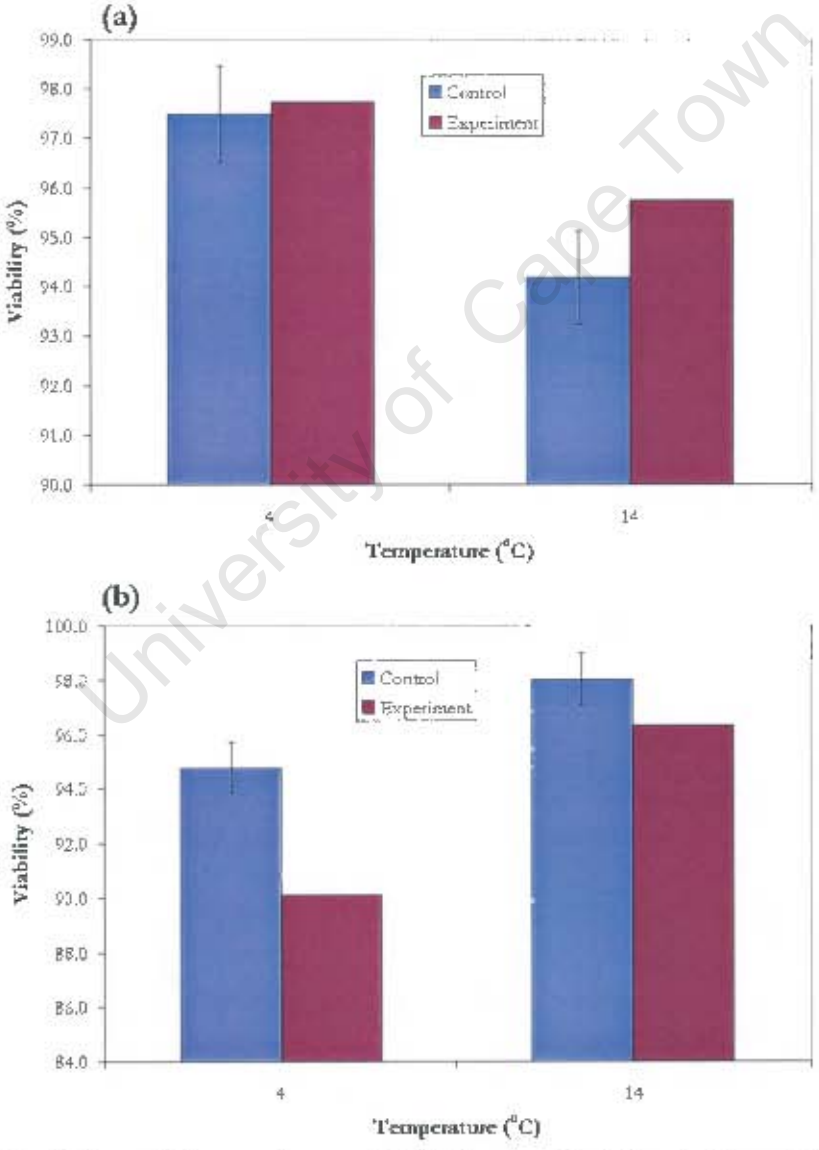

Figure 8.1: Effect of Temperature on Viability using Rushton turbine at 400 rpm for an agitation period of 8 hours.

(a) - 48.9% wet weight, (b) - 59.9% wet weight

8.3 PROTEASE ABSORBANCE

The amount of protease released was measured by a modified method by Robinson, (2001). The difference between the protease absorbance of two yeast samples are said to be statistically significant if it is greater than 0.002. In Figure 8.2a, yeast suspension of 48.9% wet weight and Rushton turbine at an agitation rate of 400 rpm (impeller tip speed of 0.72 ms^{-1}) was used and in Figure 8.2b, slightly diluted yeast suspension with a concentration of 44.0% wet weight and an agitation rate of 800 rpm (impeller tip speed of 1.44 ms^{-1}). At a suspension concentration of 48.9% wet weight and an agitation rate of 400 rpm, as the storage temperature increased from 4 to 14°C, the amount of protease released increased; an increase of 0.002 at 4°C and an increase of 0.007 at 14°C. At 44.0% wet weight and a higher agitation rate of 800 rpm, there was no significant increase in protease absorbance at 4°C (0.002) but a significant increase of 0.009 at 14°C.

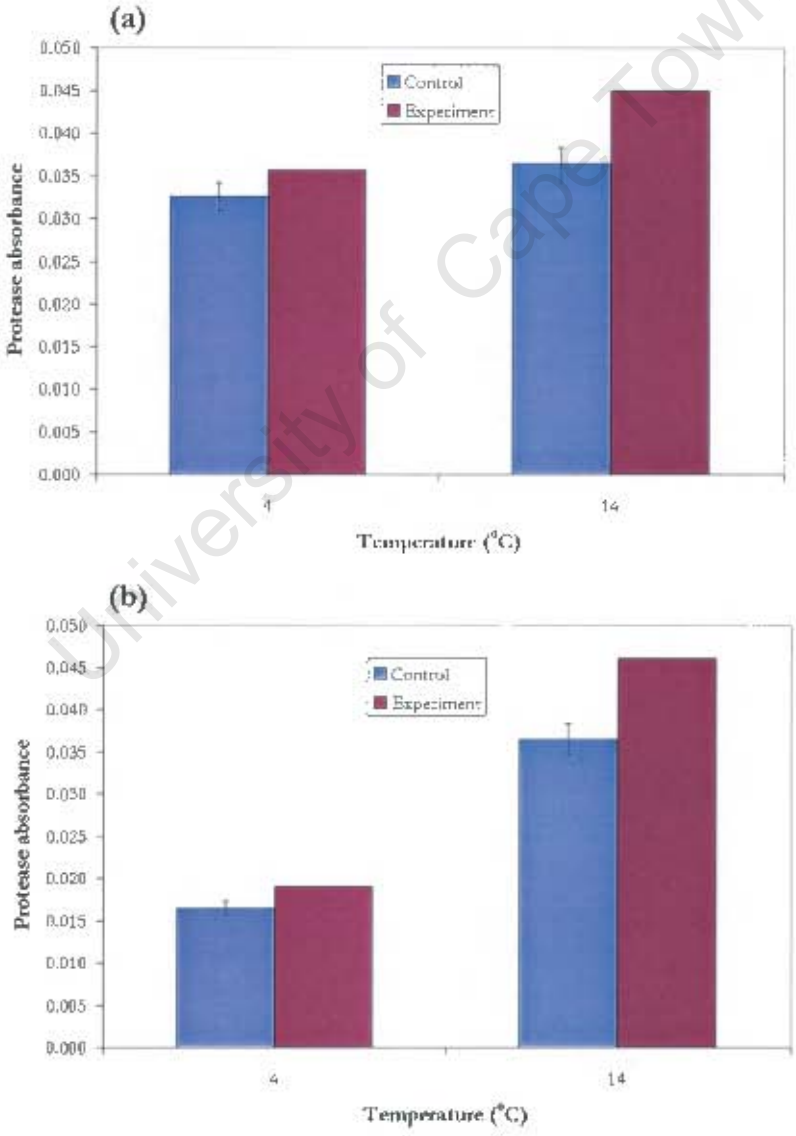

Figure 8.2: Effect of Temperature on Protease Absorbance
(a) - 48.9% wet weight & 400 rpm, (b) - 44.0 % wet weight & 800 rpm

8.4 HAZE GENERATION

The amount of haze generated was measured by the use of a particle size distribution curve obtained from the Mastersizer. A detailed methodology for quantifying the amount of haze is shown in Section 3.9.2.1. Figure 8.3 shows how the amount of haze generated changes with the storage temperature. Rushton turbine at agitation rates of 400 and 800 rpm and the yeast with a concentration of 60.7% wet weight was used to obtain the data in Figure 8.3. The duration of agitation was 8 hours. At an impeller speed of 400 rpm (impeller tip speed of 0.72 ms^{-1}), there is a slight increase (15.9%) in the amount of haze on increasing the vessel temperature from 4 to 14°C. However, an impeller speed of 800 rpm (impeller tip speed of 1.44 ms^{-1}) and increasing the vessel temperature from 4 to 14°C led to 61.5% increase in the amount of haze.

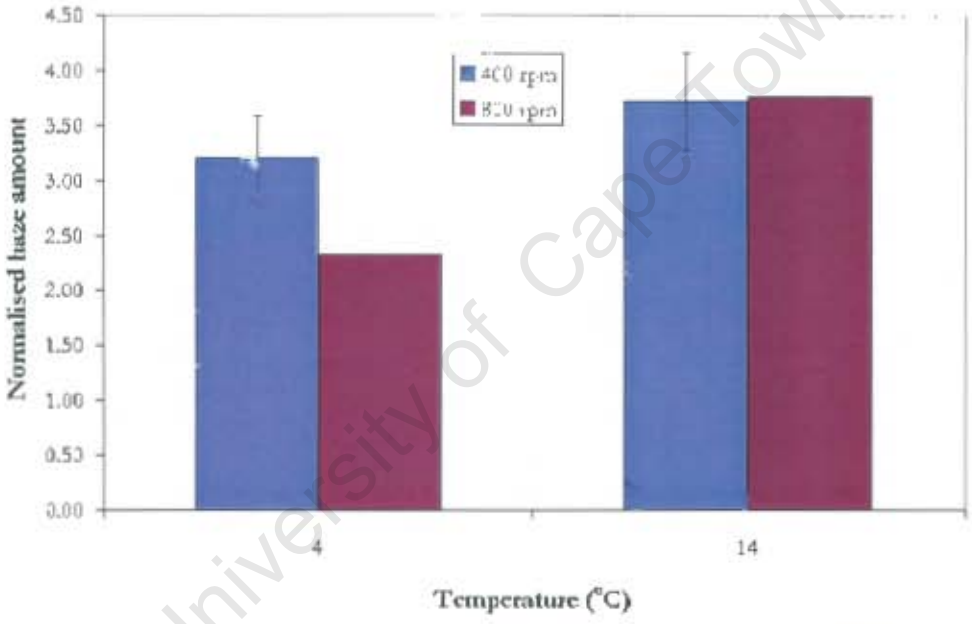

Figure 8.3: Effect of temperature on the amount of haze generated using Rushton turbine and yeast concentration of 60.7% wet weight for 8 hours

8.5 VITALITY

The data shown in Tables 8.1 and 8.2 were obtained by agitating yeast suspension with a concentration of 46.1% wet weight with Rushton turbine for a duration of 8 hours. The growth rate increases as the vessel temperature is increased from 4 to 14°C although the biomass yield does not change and neither does the carbon dioxide yield. However, the ratio of the initial to final cell concentration increases significantly (Table 8.1). The substrate utilization rate increases as the temperature increases while the carbon dioxide formation rate decreases slightly (Table 8.2).

Table 8.1: Growth parameters as a function of temperature

Temperature (°C)	Speed (rpm)	μ (h^{-1})	$Y_{X/S}$ (10^9 cells/g)	X_f/X_i	$Y_{CO2/S}$ ($ngCO_2$/cells.g)
4	400	0.05	0.07	1.21	0.51
	800	0.02	0.08	1.55	0.30
14	400	0.03	0.07	1.84	0.25
	800	0.09	0.09	2.49	0.35
Standard Deviation		±0.02	±0.01	±0.22	±0.06

Table 8.2: Substrate utilization and carbon dioxide formation rates as a function of temperature

Temperature (°C)	Speed (rpm)	q_S (ng/h.cells)	q_{CO2} (ng/h.cells)
4	400	0.19	0.22
	800	0.34	0.14
14	400	0.54	0.12
	800	0.68	0.17
Standard Deviation		±0.08	±0.03

8.6 Discussion And Conclusions

In this chapter the effect of storage temperature on yeast quality is investigated. Cropped yeast was subjected to mechanical stress by agitating with Rushton turbine at impeller speeds of either 400 or 800 rpm and vessel temperatures of 4 and 14°C. The yeast suspensions used were not diluted and their concentrations varied between 44.0 and 60.7% wet weight. After the yeast suspension had been subjected to mechanical stress, it was then assayed for protease release, viability, vitality and haze present. As the storage temperature is decreased from 4 to 14°C, the yeast viability decreased and the amount of haze generated increased. In the small-scale fermentation experiments used to quantify the vitality of the agitated yeast, it can be concluded that as the storage temperature decreased, the growth rates, the substrate utilisation rates and the change in cell concentration (X_f/X_i) decreased. The yield of carbon dioxide on substrate and the carbon dioxide formation rates increased while the yield of biomass on substrate did not change. Finally, the amount of protease released decreased as the storage temperature increased. These results except for the protease results are similar to those obtained by Nkosi (2001), who demonstrated that the yeast subjected to temperature stress by rapid cooling are more susceptible to damage to the cell envelope and loss of viability. Nkosi (2001) observed that temperature stress to yeast cells is dependent on the rate of cooling. In this study, the yeast suspension obtained from SABMiller Brewery in Newlands arrive in the laboratory at 15°C and would be cooled down to 4°C in the stirred tank reactor in 20 minutes (depending on the agitation rate used), giving an average cooling rate of 0.5°C/min. This cooling rate is higher than that recommended by Nkosi (2001), who suggested a cooling rate

of 1°C/h. Walker (1998) also suggested that low temperature stress can lead to formation of leaky membranes (as indicated by haze), vacuolar damage because of the shrinkage of the yeast and cell death (loss of viability). The history (generation number, cooling rates) of the yeast suspension prior to mechanical stress is very important. This is evident in the cooling rates used prior to agitation. The results show that the yeast cells are more susceptible to damage when cooled at a rapid rate and over a wider range of temperature (15 to 4°C as opposed to 15 to 14°C).

CHAPTER 9: CONCLUSIONS AND RECOMMENDATIONS

9.1 CONCLUSIONS

The lager fermentation process at SABMiller involves the use of yeasts to convert sugars to ethanol (SAB, 1993). After fermentation is complete, the *Saccharomyces carlbergensis* settles while *Saccharomyces cerevisiae* which flocculates, is stored in the yeast collection vessel (YCV). In the YCV, the yeast suspension is agitated and stored under a nitrogen headspace at 4°C for between 4 and 48 hours. During storage in the YCV, the yeast is subjected to mechanical stress through agitation, and physiological stress through temperature fluctuations, exposure to oxygen and ethanol and possible nutrient limitations. Even though yeast cells are quite robust and not easily damaged by mechanical stress (Walker, 1998), the extent of damage is highly dependent on the intensity and exposure time to such stresses. The following can be used as indicators of yeast quality: yeast viability, yeast vitality, integrity of the cell envelope, flocculation and sedimentation characteristics and surface properties. In this study, the biological response of *Saccharomyces cerevisiae* to mechanical stress by agitation was investigated by modelling the YCV on a laboratory scale.

Biological response of cells to mechanical stress was quantified by looking at yeast viability, vitality and the integrity of the cell envelope. Also, the manner by which mechanical stress by agitation can affect yeast quality was hypothesised. Agitation can lead first to minor wall damage and later to cell membrane damage, which can be quantified by yeast viability and the amount of extracellular protease released. Further exposure or an increase in the intensity of mechanical stress can then lead to compromise in the cell wall integrity which can be measured by the amount of haze generated. Loss of viability may affect subsequent yeast performance (overall yeast quality). Yeast viability was measured by the methylene blue staining technique, extracellular proteases by the method by Mochaba *et al.* (1993) and yeast vitality by performing small-scale fermentations on mechanically stressed yeast.

The extent of damage to yeast cells by mechanical damage is dependent on the magnitude of agitation (turbulence), the exposure time as well as the number of cells (concentration of yeast suspension) that are exposed to mechanical stress. The concentration of the suspension affects the rheology of the fluid which in turn affects the intensity of agitation. In order to vary the magnitude of mechanical stress, the contents of the experimental STR was agitated at different agitation rates with the use of two types of impellers. The exposure time of the yeast cells to agitation was also varied between 4 and 24 hours. The extent of mixing and the rheology of the yeast were determined at different yeast concentrations, agitation rates and impeller types. The optimum agitation rate, exposure time, storage temperature and yeast concentration were then identified.

The t-test, f-test and ANOVA were the statistical tools used to analyse the results obtained from the investigation into the effect of agitation on yeast quality. Yeast suspensions collected on different days were tested for significance by the use of the ANOVA and there was no significant difference in the time profile of the viabilities of the yeast suspension collected on different days. Furthermore, there were no observed variations in viability as a function of time for yeast suspensions of different generations. The coefficients of variance for the different analytical methods were calculated and they all fell within 15.0% of the mean. The reproducibility of all the methods was good although the method by which the amount of carbon dioxide formed is measured requires improvement. Finally, the mixing data were found to be less reproducible the higher the cell concentration and the more the number of tracer additions.

In this study, the rheology of the yeast suspension was determined at different concentrations and it was found that the rheology of yeast suspensions is dependent on the suspension concentration. In the lower concentration ranges (< 20% wet weight), the rheology of the yeast suspension was found to be Newtonian. In the concentration ranges above 20% wet weight, the rheology of the yeast suspension was shown to be non-Newtonian, more specifically approximated as pseudoplastic. The results obtained in this study is similar to the findings of Lenoël *et al*, (1987) who found that yeast suspension exhibits a pseudo-plastic rheology at dry matter content greater than 40% pressed yeast (as estimated by the Buchner

test) and thinner yeast suspensions have a Newtonian rheology. Reuss *et al.* (1979) and Rudiš *et al.* (1976) also reported similar findings. The consistency index, K showed an exponential relationship with the suspension concentration. The rheograms were fitted with different models and at yeast concentrations between 20 and 44.0% wet weight; the power law model fitted the data whereas at higher yeast concentrations (58.0 to 66.3% wet weight), the Herschel-Bulkley model gave the best fit. Yeast suspensions at higher concentrations (58.0 to 66.3% wet weight) fitted the Herschel-Bulkley model because they had yield stresses that needed to be overcome before any fluid movement could take place. Reuss *et al.* (1979) obtained a hyperbolic relationship between volume fraction of *Saccharomyces cerevisiae* suspension, its packing factor (a function of osmotic pressure) and its relative viscosity (ratio of suspension viscosity and supernatant viscosity). The relationship between the apparent viscosity and suspension concentration was found to be exponential because the R^2 values of the regression lines were high (< 0.95) This result is different from that obtained by Rudiš *et al.* (1976) who reported a cubic relationship between apparent viscosity and yeast suspension concentration. The reason for the difference is probably due to the range of yeast concentrations (between 0 and 10% wet weight) investigated by Rudiš *et al.* (1976). It is established from the research that the nature of the rheology of the yeast suspension as well as magnitude of the apparent viscosity is a function of its concentration.

A relationship between the rheology of the yeast suspension and mixing time was established by investigating the effect of suspension concentration on mixing time. As the yeast concentration is increased from 30 to 40% wet weight, there is a small increase in the mixing time whereas an increase in concentration from 40 to 50% wet weight resulted in almost a 6-fold increase in mixing time. Hence, the change in mixing time can be correlated largely with apparent yeast viscosity within these concentration ranges. An investigation into the effect of agitation rate on mixing was conducted over a range of suspension concentrations. At suspension concentrations below 32% wet weight, mixing time is independent of agitation rate. Here, on increasing the agitation rate 4 fold, mixing times recorded were within 8% of the average, lying within the coefficient of variance of analysis. However, as the cell concentration exceeded 37% wet weight, mixing time became increasingly dependent on agitation rate. Of the two impeller types, the Rushton impeller gives better mixing than the pitched blade. This is because the Rushton impeller dissipates more power than the pitched blade thereby leading to an increase in the energy available for mixing. Further, the Rushton impeller gives radial mixing and has good viscosity tolerance which is useful when mixing highly viscous fluids (Rushton *et al*, 1950b).

The effect of the intensity of agitation on yeast quality was investigated by either varying the agitation rate or impeller type. Intensity of agitation is a function of the geometry, type and speed of the impeller used. Impeller geometry was eliminated from the factors being investigated by using impellers of standard geometry. A range of impeller speeds from 200 to 1200 rpm and two types of impellers (Rushton turbine and 45° pitched blade) were used to alter the intensity of agitation. It was concluded that the more intense the agitation, the more pronounced the loss of viability and loss of membrane integrity. These results are similar to

alter the intensity of agitation. It was concluded that the more intense the agitation, the more pronounced the loss of viability and loss of membrane integrity. These results are similar to those obtained by McCaig and Bendiak (1985a) in which they observed a reduction in the viability of pitching yeast agitated at a rate of 0.72 ms^{-1} at 1°C for 5 days. Subsequent fermentations become less successful if the impeller speed is increased above 400 rpm and if Rushton turbine is used at the higher speed settings (>400 rpm). The amount of haze released into suspension has a peak at 600 rpm. The result obtained in this study is different from that obtained by Lewis and Poerwantaro (1991) who concluded that the duration of agitation and not the intensity was the only factor that contributed to an increase in the amount of haze released. The amount of haze released in their study was independent of the intensity of agitation and the temperature.

The effect of the duration of agitation on yeast quality was conducted by agitating the yeast suspension for a period of 24 hours. It was observed that the longer the period of agitation, the more pronounced the loss of yeast quality and this is highly dependent on the concentration of the yeast suspension. These results are similar to those obtained by Lewis and Poerwantaro, (1991). McCaig and Bendiak (1985b) also stated that although the long duration of yeast storage affects its viability, it does not affect subsequent fermentations. These results are in contrast with those obtained in this study, in that the growth rates of yeast which had been stressed by intense agitation and subjected to long durations of agitation were less than the growth rates of yeast that had not been agitated or agitated for a shorter period.

Partially diluted and undiluted yeast suspensions were subjected to mechanical stress in order to determine the effect of dilution of the yeast suspension during storage on yeast quality. It is clearly seen from the results obtained that the yeast quality deteriorates as the suspension concentration of the storage phase increases. The loss of yeast quality can be attributed to an increase in the number of cells present in an aliquot of suspension as well as the fact that mixing becomes more difficult as the concentration increases. The presence of ethanol in the yeast suspension can induce heat shock proteins and enhance cell ageing (Walker, 1998). On diluting the yeast suspension, the concentration of ethanol is reduced and hence, a reduction in possible ethanol stress.

The effect of storage temperature on yeast quality was investigated by storing yeast in the experimental STR at 4 and 14°C and agitating the vessel contents with Rushton turbine at 400 and 800 rpm. As the storage temperature is decreased from 4 to 14°C, the yeast viability decreased and the amount of haze generated increased. Yeast growth and metabolic activity were also negatively affected by temperature decrease. Finally, the amount of protease released decreased as the storage temperature increased. These results except for the protease results are similar to those obtained by Nkosi (2001), who demonstrated that the yeast subjected to temperature stress by rapid cooling are more susceptible to damage to the cell envelope and loss of viability. Nkosi (2001) observed that temperature stress to yeast cells is dependent on the rate of cooling. The cooling rate found in the experimental STR was

stress could lead to formation of leaky membranes (as indicated by haze), vacuolar damage because of the shrinkage of the yeast and cell death (loss of viability). The history of the yeast suspension prior to mechanical stress is very important, as yeast cells that have been previously stressed are more susceptible to damage. The results also show that the yeast cells are more susceptible to damage when cooled at a rapid rate and over a wider range of temperature.

Changing the yeast suspension concentration resulted in a maximum decrease in viability of 5.8%, an increase in protease released of 0.031 absorbance units, an increase in the amount of haze generated of 2.8 and a decrease in growth rate of $0.07h^{-1}$. Varying the storage temperature from 4 to 14°C led to a maximum decrease of 3.2% in yeast viability, an increase of 0.009 absorbance units of protease released, an increase of 1.0 in the amount of haze generated and a maximum decrease of $0.03\ h^{-1}$ in the growth rate of small-scale fermentations. In changing the agitation rate, loss of yeast quality is highly dependent on the suspension concentration. Comparing the interaction between the operating variables (suspension concentration, temperature and agitation rate), the change in yeast suspension concentration has the most significant effect on the loss of yeast quality

9.2 RECOMMENDATIONS

From the results obtained from this study, the optimum agitation rate and exposure time above which mechanical stress to stationary phase yeast may cause damage was postulated. The optimum agitation rate was found to be 400 rpm (impeller tip speed of $1.44\ ms^{-1}$) and exposure time should not be greater than 8 hours. In the brewery YCV, the equivalent agitation rate is 48.2 rpm. This agitation rate would be sufficiently intense to effect good mixing and homogeneity in the YCV, if the yeast suspension is diluted to between 30 and 40% wet weight (consistency).

This study was limited to using the Rushton turbine and the 45° pitched blade impellers. Future work should focus on using complex impellers such as the helical ribbon. These impellers are more efficient for homogenising pseudoplastic fluids but might actually cause more damage to the cells due to the intensity of mixing and an increase in the extent of mechanical stress. Other yeast quality indicators such as the cell surface properties and flocculation and sedimentation characteristics should be investigated as well.

REFERENCES

Allen, D.G. and Robinson, C.W. (1990), "Measurement of rheological properties of filamentous fermentation broths". *Chemical Engineering Science* 45(1), 37-48

Amory, D. E.; Rouxhet, P. G. (1988), "Surface properties of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergenis*". *Biochemica et Biophysica Acta*, 61-70.

Basson, L. (1996), "Loss of yeast quality during mechanical handling in a brewery: an investigation of cropping". MSc (Eng) Thesis, Chemical Engineering, University of Cape Town.

Blanch, H.W. and Clark, D.S. (1997). Biochemical Engineering, Marcel Dekker Inc., New York

Birch, R.S. and Walker, G.M. (2000), "Influence of magnesium ions on heat shock and ethanol stress responses in *Saccharomyces cerevisiae*". *Enzyme and Microbial Technology*. 26, 678- 687

Boughton, R.A. (1983), "Are you getting the "best" out of your yeast?" *The Brewer*. 69, 260-264.

Brown N.J., (1998), "The effect of non-biological particulates on microbial cell disruption in the slurry reactor" PhD (Eng) Thesis, Chemical Engineering, University of Cape Town.

Bryant J. (1977), "The characterization of mixing in fermenters". *Advances in Biochemical Engineering*. 5, 101.

Coulson, J.M., Richardson, J.F., Backhurst, J.R., Harker, J.H. (1999) Coulson & Richardson's Chemical Engineering. Volume 1. Butterworth Heinemann, Oxford.

Chisti, Y. (1999). Sheer sensitivity. In: Flickinger, M.C. and Drew, S.W. (eds). Encyclopaedia of Bioprocess Technology – fermentation, bio catalysis and bioseperation. Volume 5. John Wiley and Sons, Inc., New York.

D'Amore, T., Panchal, C.J., Russell, I. and Stewart, G.G. (1990), "A study of ethanol tolerance in yeast". *Critical Reviews in Biotechnology*. 9, 287- 304

D'Amore, T., Crumplen, R. and Stewart, G.G. (1991), "The involvement of trehalose in yeast stress tolerance". *Journal of Industrial Microbiology*. 7, 191-196

D'Hartcourt, O., Smart, K.A. (1999), "Measurement of brewer's yeast flocculation". *Journal of the American Society of Brewing Chemists*, 57(4), 123-128.

Daoud, I.S. and Searle, B.A. (1986), "Yeast vitality and fermentation performance". *European Brewing Convention Monograph XII Symposium on Brewing Yeast 1986*. 108-121

Doran P.M. (1995). Bioprocess Engineering Principles. Academic Press, London. Chapter 7.

Dunlop, E.H., Pradyumna, K.N., Rosenberg, M.Z. (1994). "Effect of fluid shear forces on plant cell suspensions". *Chemical Engineering Science*, 49(14), 2263-2276

Estruch, F. (2000). "Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast". *FEM Microbiology Reviews,* 24, 469- 486

Fargher, J. and Smith, N.A. (1995), "Evidence of cold shock sensitivity in brewing yeast strains". *European Brewing Convention.* 40, 14- 22

Fasano, J. and Penney, W. (1991), "Avoid blending mix-ups". *Chemical Engineering Progress.* 57- 63.

Fisher, S., Schafer, K., Sommer, K. (2001), "Separation performance of a centrifuge for wheat beer separation". *Brauwelt International,* 40, 14-22.

Guérin, P., Carreau, P.J., Patterson, W.I., Paris, J. (1984), " Characterisation of helical impellers by circulation times". *The Canadian Journal of Chemical Engineering.* 62, 301-309

Harrison, S.T.L., Raja, S., Sissing, A., Nemati, M. (2003), "Identifying and quantifying biological response of *Sulfolubus* to high pulp densities in the slurry bioleach reactor". *International Mineral Processing Convention, Cape Town, September, 2003.*

Immunity Information Network, "Beta Glucan and Beta-1,3-D Glucan". http://www9.ocn.ne.jp/~immunet/e11.html. Last viewed in December, 2003.

Iwahashi, H., Obuchi, K., Fujii, S., Komatsu, Y. (1995), "The correlative evidence suggesting that trehalose stabilizes membrane structure in the yeast *Saccharomyces cerevisiae*". *Cellular and Molecular Biology.* 38, 763-769.

Jones R.P. (1987), "Measure of yeast death and deactivation and their meaning". *Process Biotechnology,* 22, 118-128.

Khang, S. and Levenspiel, O. (1976), " The mixing rate number for agitator stirred tanks". *Chemical Engineering,* 83, 141- 143.

King, L.M., Schisler, D.O., Ruocco, J.J. (1981), "Epifluorescent method for detection of nonviable yeast". *Journal of the American Society of Brewing Chemists.* 39(2), 52-54.

Knudsen, F.B. (1985), "Fermentation variables and their control". *Journal of the American Society of Brewing Chemists.* 43(2), 91-95.

Komatsu, Y.; Kaul, S. C.; Iwahashi, H.; Obuchi, K. (1990), "Do heat shock proteins provide protection against freezing?". *FEMS Microbiology Letters* . 17, 159-162

Kouda, T.; Yano, H.; Yoshinaga, F.; Kaminoyama, M.; Kamiwano, M. (1996), "Characterisation of non-Newtonian behaviour during mixing of bacterial cellulose in a bioreactor". *Journal of Fermentation and Bioengineering,* 82(4), 382-386

Lamaignére, V., (2002), "Effects of hydrodynamic stress on growing *Saccharomyces cerevisiae* in a slurry reactor". MSc (Eng) Thesis, Chemical Engineering, University of Cape Town

Lee, S.S., Robinson, F.M., Wang, H.Y. (1981), "Rapid determination of yeast viability". *Biotechnology and Bioengineering Symposium,* 11, 641-649.

Lenoël, M., Meunier, J.-P., Midoux, N. (1987), "Improved system for stabilizing yeast fermenting power during storage". *EBC Congress, Madrid.* 425-432

Lentini, A. (1993), "A review of the various methods available for monitoring the physiological status of yeast: Yeast viability and vitality". *Ferment.* 6(5) 321-327

Lewis, M.J. and Poerwantaro, W.M. (1991), "Release of haze material from the cell walls of agitated yeast". *Journal of the American Society of Brewing Chemists.* 49(2), 43-46.

Martens, F.B., Egberts, G.T.C., Kempers, J., Robles de Medina, M.H.L., Welten, H.G.J. (1986), "Yeast storage methods and their effects on fermentation". *European Brewing Convention Monograph XII, Brewers' Yeast, Vuoranta (Helsinki), November, 1986.* 95-105

McCabe, W.L., Smith, J.C., Harriott, P. (2001). Unit Operations of Chemical Engineering. 6th edition. McGraw-Hill. New York.

McCaig, R. and Bendiak, D.S. (1985a), "Yeast handling studies. I. Agitation of stored pitched yeast". *Journal of the American Society of Brewing Chemists.* 43(2), 114-118.

McCaig, R. and Bendiak, D.S. (1985b), "Yeast handling studies. II. Temperature of storage of pitched yeast". *Journal of the American Society of Brewing Chemists.* 43(2), 119-122.

McCaig, R. (1990), "Evaluation of fluorescent dye 1-Anilino-8-naphthalene sulfonic acid for yeast viability determination". *Journal of the American Society of Brewing Chemists.* 48(1), 22-25

Metzner, A.B. and Otto R.E. (1957), "Agitation of non-Newtonian fluids". *A.I.Ch.E Journal.* 3(1) 3-10

Mizoguchi, H., and Hara, S., (1997), "Ethanol-induced alterations in lipid composition of *Saccharomyces cerevisiae* in the presence of exogenous fatty acid". *Journal of Fermentation and Bioengineering.* 83, 12-16

Mochaba, F., Touline, P.A., Axcell, B. (1993), "Resorufin-labelled casein as a substrate for protease activity in beer", *EBC Congress, Oslo*, 533-541.

Moo-Young, M., Tichar, K., Dullien, F.A.L. (1972). "The Blending efficiencies of some impellers in batch mixing". *AIChE Journal.* 18(1) 178-182

Murray, C.R., Barich, T., Taylor, D. (1984), "The Effect of Yeast storage conditions on subsequent Fermentations". *MBAA Technical Quarterly.* 21(4), 189-194.

Napier-Munn, T.J. (1994). An *Introduction to* Comparative Statistics and Experimental Design *for Minerals Engineers.* Course notes, 2nd edition. University of Queensland

Nishikawa, N. and Nomura, B. (1974), "Numerical evaluation of yeast deterioration caused by repeated fermentation". *Proceeding of the 13th Convention of the Institute of Brewing (New Zealand and Australian Section).* Surface Paradise. 105-112

Nkosi, J., (2001), "The effect of cooling on brewers' yeast quality". MSc (Eng) Thesis, Chemical Engineering, University of Cape Town

O'Connor-Cox, E. (1994), " I. Yeast Nutrition: Do's and Don'ts for AJL2036". Current Impressions, SAB Brewing Research & Development Technical Bulletin, 1-19.

Oldshue, J.Y. (1983). Fluid mixing technology. McGraw-Hill. New York

Ormrod, I.H.L., Lalor, E.F., Sharpe, F.R. (1991), "The release of yeast proteolytic enzymes into beer". *Journal of the Institute of Brewing*, 97, 441-443.

Perry, R.H., Green, D.W., Maloney, J.O. (1998). "Chapter 18: Liquid Solid Operations" In Perry's Chemical Engineers' Handbook. 8th edition, McGraw-Hill, Singapore.

Pickerell, A.T.W., Hwang, A., Axcell, B.C. (1991), " Impact of yeast-handling procedures on beer flavour development during fermentation". *Journal of the American Society of Brewing Chemists*, 49(2) 87-92

Quain, D.E. (1988), "Studies on yeast physiology-impact on fermentation performance and product quality". *Journal of the Institute of Brewing*, 95, 315-323.

Reuss, M., Josic, D., Popovic, M., Bronn, W.K. (1979). "Viscosity of yeast suspensions". *European Journal of Applied Microbiology and Biotechnology*. (8) 167-175.

Roessler, J.G. (1968), "Yeast Management in the Brewery: Yeast handling treatment Part II" *Brewer's Digest*, 94-115

Robinson, A. (2001). "Mechanical handling effects on Brewers' yeast". PhD Thesis. University of Cape Town, South Africa.

Rudiš, M., Jezdinský, V., Štěrbáček, Z. (1976). " Physical properties of microbial suspensions. II: Properties of microbial suspensions and their supernatants during fermentation conditions". *Folia Microbiol.* 21 128- 133.

Rushton, J.H., Costich, E.W., Everett, H.J. (1950a). "Power characteristics of mixing impellers. Part I. *Chemical Engineering Progress.* 46(8) 395-404.

Rushton, J.H., Costich, E.W., Everett H.J. (1950b). "Power characteristics of mixing impellers. Part II. *Chemical Engineering Progress.* 46(9) 467-476

SAB (1993). A Booklet of Good Brewing Practise. South African Breweries (Beer Division). In-house publication.

Schur, F. (1990). "Yeast treatment and beer quality". *Brau Rundsch*, 101(9), 147-151.

Sall, C.J., Seipp, J.F., Pringle, A.T. (1988), "Changes in Brewer's yeast during storage and the effect of these changes on subsequent fermentation performances". *Journal of the American Society of Brewing Chemists.* 46(2), 23-25

Siebert, K.J., Stenroos, L.E., Reid, D.S. (1981), "Characterization of amorphous-particle haze". *Journal of the American Society of Brewing Chemists.* 39(1), 1-11

Sissing, A.A. (2002), "An investigation into the effect of solid particulate phase on the bioleaching performance of *Sulfolobus metallicus*". MSc (Eng) Thesis, Chemical Engineering, University of Cape Town

Smart, K.A., Boulton, C.A., Hinchliffe, E., Molzahn, S. (1995), "Effect of physiological stress on the surface properties of brewing yeasts". ***Journal of the American Society of Brewing Chemists,*** 53(1), 33-38.

Smart, K.A, Whisker, S. (1996), "Effect of serial repitching on the fermentation properties and condition of brewing yeast". ***Journal of the American Society of Brewing Chemists,*** 54 (1), 41-44

Smart K.A., Chambers K.M., Lambert I., Jenkins C. and Smart C.A. (1999). "Use of methylene violet staining procedures to determine yeast viability and vitality. ***Journal of the American Society of Brewing Chemists,*** 57(1), 18-23

Tuite, M.F., Oliver, S.G. (1991). "*Saccharomyces*". Biotechnology Handbooks. Vol. 4, Plenum Press, New York.

Walker, G., (1998). Yeast Physiology and Biotechnology. John Wiley and Sons, New York.

Wang, N.S, "Experiment No. 4A: Glucose Assay by dinitrosalicylic acid colorimetric method". http://www.glue.umd.edu/~nsw/ench485/lab4a.htm. Last viewed on 17th October 2003.

Zlokarnik, M., and Strasse, G. (2002), "Stirring" In Ullmann's Encyclopedia of Industrial Chemistry. 6th edition, Vol. 34. Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim.

APPENDIX A: ASSAY REAGENTS

A.1 METHYLENE BLUE STAINING ASSAY

0.025% Methylene Blue

0.25g/l methylene blue
9g/l NaCl
0.42g/l KCl
0.48g/l $CaCl_2.6H_2O$
0.2g/l $NaHCO_3$
10g/l glucose
in distilled water

Phosphate buffer solution (PBS)

0.1342g/l KCl
8.0647g/l NaCl
0.245g/l KH_2PO_4
3.769g/l $Na_2HPO_4.12H_2O$
in distilled water

2mM EDTA

0.83g/l EDTA-tetra sodium salt
in distilled water

A.2 PROTEASE ASSAY

Substrate Solution

15mg Casein, resorufin-labelled (Boehringer Mannheim, Universal Protease substrate Cat. No 1080733)
Add 3.75ml deionised, distilled water to contents
Put aliquots of 50µl into eppendorf tubes
Store in deep freeze

Incubation buffer

Tris-HCl Buffer
0.2M Tris-HCl, pH 7.8, 0.02M $CaCl_2$
in distilled water

Stop reagent

Trichloroacetic acid
5% w/v in deionised distilled water

Assay Buffer

Tris-HCl
0.5M Tris-HCl, pH 8.8
in distilled water

A.3 SMALL SCALE FERMENTATION

A.3.1 GROWTH MEDIUM

MYPG Media

3g/l malt extract
3g/l yeast extract
5g/l peptone
10g/l glucose
in distilled water

A.3.2 GLUCOSE ASSAY

1% Dinitrosalicylic (DNS) Acid Reagent solution

10g/l Dinitrosalicylic acid
2g/l Phenol
0.5g/l Sodium sulfite
10g/l Sodium hydroxide
in distilled water

40% Potassium tartrate (Rochelle salt) solution

40g/l potassium tartrate
in distilled water.

APPENDIX B: ASSAY METHODS

B.1 Methylene Blue Assay

B.1.1 METHOD

This method is that proposed by Lee *et al.*, 1981. The staining solution is detailed in Appendix A1. One part of yeast slurry was diluted with 3 – 4 parts phosphate buffer solution (PBS) depending on the suspension concentration, and 9 parts EDTA whose function is to de-flocculate the cells. This solution was further diluted by adding 1 part of the solution above to 9 parts of methylene blue solution. The suspension is left to stand for about a minute before counting. Place a drop of the resulting solution on a haemocytometer and count the number of blue cells and the total number of cells under a brightfield microscope at 400X magnification. Viability is then calculated as thus:

$$Viability = \frac{Total\ number\ of\ cells - Number\ of\ blue\ cells}{Total\ number\ of\ cells} * 100\% \quad \text{Equation B1}$$

B.1.2 REPRODUCIBILITY

The standard deviation of the viability measurements obtained from counting a samples 5 times is 0.95% and a coefficient variance of 1.02%.

Table B.1: Typical viability results

Sample No.	Viability (%)
1	94.0
2	93.2
3	92.9
4	93.4
5	95.2
Average	93.7
Standard deviation	0.95
Coefficient of variance (%)	1.02

B.2 Direct Microscopic cell counting

B.2.1 METHOD

The diluted suspension in PBS, EDTA and methylene blue solution (Appendix A1) is placed on the haemocytometer and covered with a cover slip. The counting chamber is viewed under a brighfield microscopy at X400 magnification. The chamber consists of 25 blocks with each block consisting of 16 smaller squares. The depth of the chamber is 0.1mm and each block covers a $0.0001mm^2$ area. Cells are counted in all 25 blocks ensuring that an average of 200 cells are counted in order to have a statistically significant count. Cells having less than 50% of their size enclosed in the perimeter are ignored (cell A in Figure B1), while cells with more than 50% enclosed within the perimeter lines are counted (cell B in Figure B1). In counting budding cells, buds that are more than half the size of the mother cell (Cell C in Figure B1) are regarded as fully grown cells and counted as such. However, buds that are smaller than half the size of the mother cell (Cell D in Figure B1) are ignored. Thus, cell concentrations and viabilities are calculated by using equations B1 and B2.

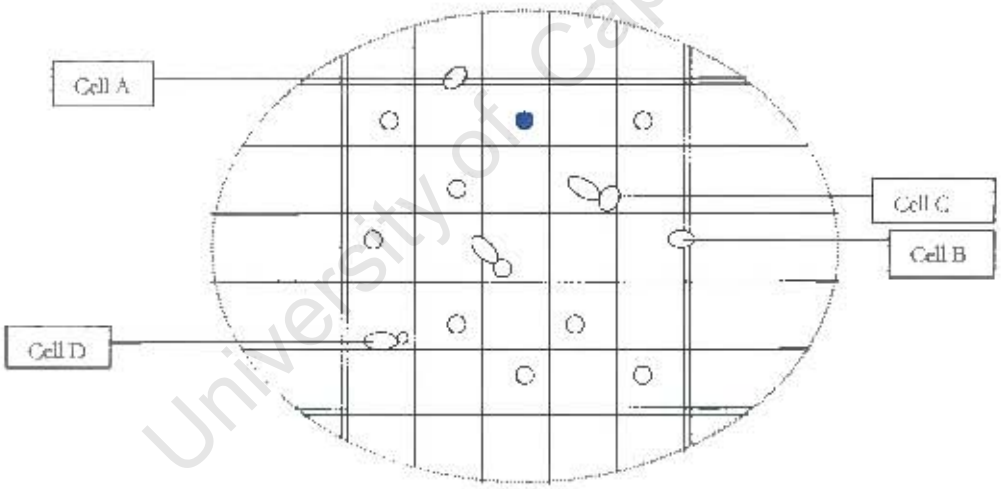

Figure B1: Direct Cell count

$$Cell\ concentration(cells/ml) = \frac{Cell\ count \times Dilution\ factor \times 1000}{0.0025 \times 0.1} \quad \text{------- Equation B2}$$

B.2.2 REPRODUCIBILITY

The standard deviation for cell concentration on four repeated samples is 0.08 * 10^6 cells/ml and a coefficient of variance is 4.7%.

B.3 Haze Analysis

The yeast suspension was centrifuged at 3000rpm at 4°C for 5 minutes in the Beckman J-25 Avanti centrifuge to remove the bulk of the yeast. The supernatant obtained was then analysed for haze in the Malvern mastersizer. The settings used in the mastersizer is 50% of the maximum pump capacity, 50% of the maximum stirrer speed and 20% of the maximum ultrasonic power possible. The density of the yeast suspension was set at 1.000g/cm^3. The amount of obscuration needed for good results is between 8 and 14%. In the mastersizer, a size distribution of the sample is obtained (Number or Volume % as a function of the particle size in μm. Haze material was classified as particles with diameter less than 2μm. A full description of how haze is calculated is shown in Section 3.9.3.1.

B.3.1 REPRODUCIBILITY

The reproducibility of this method is well treated in Section 4.2.4. The coefficient of variance of the average particle size of four replicates is 2.4% while the coefficient of variance of the amount of haze in a given sample is 13.8%.

B.4 Extracellular Protease Assay

B.4.1 METHOD

The steps necessary to perform the extracellular protease assay are listed below.

1. Centrifuge the yeast suspension in an Eppendorf microfuge at 10 000rpm for 5 min.
2. Decant supernatant into a clean Eppendorf tube.
3. Centrifuge the supernatant for 5 min
4. Decant resulting supernatant into a new Eppendorf tube.
5. Pipette 50µl substrate (Appendix A2) into fresh Eppendorf tubes (Pre-prepared).
6. Add 50µl of incubation buffer (Appendix A2) to substrate.
7. For sample add 100µl supernatant and for blank add 100µl of deionised distilled water.
8. Mix by tapping gently on the bench.
9. Incubate at 37°C for 1hr.
10. Add 480 µl Stop reagent (Appendix A2) and mix.
11. Incubate at 37°C for 10 min.
12. Centrifuge for 5 min and decant supernatant into fresh Eppendorf tube.
13. Pipette 400 µl of supernatant into 1ml microcuvettes.
14. Add 600 µl assay buffer (Appendix A2).
15. Measure absorbance at 574nm in a UV/visible spectrophotometer.

$$Protease\ Activity = A_{sample} - A_{blank} \text{ ———— B3}$$

B.4.2 REPRODUCIBILITY

The standard deviation for the protease assay was found to be 0.001 on a protease activity of 0.034.

B.5 Small Scale Fermentation

B.5.1 METHOD

A 5ml aliquot of yeast suspension is used to inoculate 60ml of MYPG media (Appendix A.3.1) in the laminar flow cabinet to avoid contamination. Each flask is sealed by the use of a rubber bung fitted with a glass tube containing silica gel. The glass tube is plugged with cotton wool which has the dual function of keeping the gel from falling into the media and keeping contaminants at bay. The function of the silica gel is to absorb any moisture evaporating from the flask. A schematic of the small-scale fermentation is shown in Figure B2. After inoculation, the flask is incubated in a 30°C shaker incubator and shaken at a speed of 150rpm. Sampling (sample size of about 2ml) was done every two hours for the first 8 hours and thereafter at 24 hours. The samples taken were analysed for biomass concentration, glucose concentration, methylene blue viability and budding index. To evaluate the amount of carbon dioxide evolved, the mass of the flask before and after sampling was recorded. Therefore any weight loss is attributed to carbon dioxide.

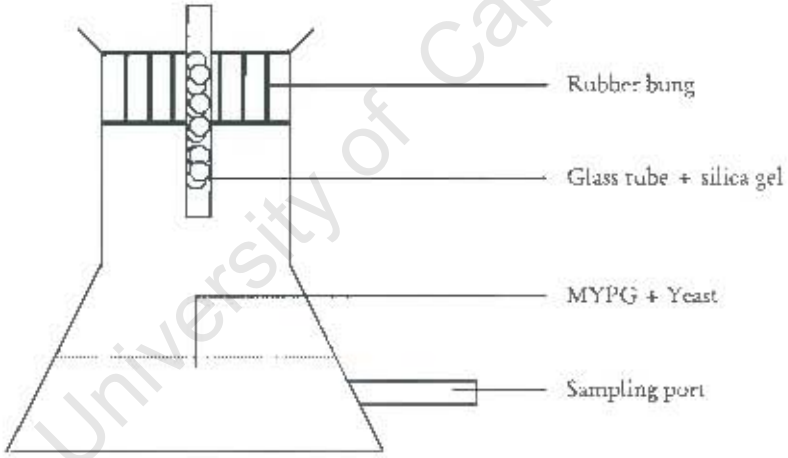

Figure B2: Set-up for small-scale fermentation

B.5.2 REPRODUCIBILITY

The reproducibility of the small-scale fermentation is reported in Session 4.2.3.2.

B.6 Glucose Assay

B.6.1 METHOD

The steps listed below were followed in order to quantify the amount of glucose in a sample.

First, a standard curve for glucose is obtained by:

1. Dissolve 0.1g of glucose in 100 ml of distilled water
2. Pipette out 0, 100, 200, 400, 500, 600, 800, 900 µl of the above solution into test tubes and make them up to 1ml with distilled water.
3. Add 600µl of DNS reagent (Appendix A.3.2) to 600µl of glucose sample into a test tube.
4. Cover the test tube with parafilm.
5. Heat the mixture at 90°C for 5 min to develop a red-brown colour
6. Pipette 200µl of the Rochelle salt and add it to the mixture to stabilise the colour
7. Cool to room temperature in a cold water bath
8. Record the absorbance at 574nm with the use of a spectrophotometer.
9. Obtain a linear regression of the glucose concentration as a function of the absorbance (Standard curve). Figure B1 shows a typical calibration curve.

Linear regression:

$$Glucose\ concentration(g/l) = slope * absorbance + constant \quad \text{----- Equation B4}$$

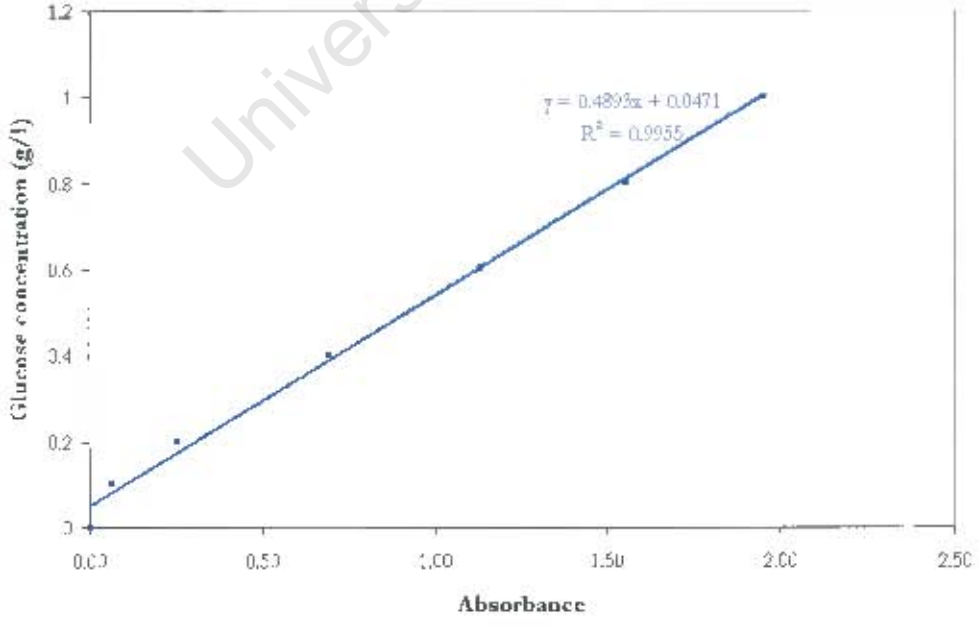

Figure B1: Typical standard curve for determination of glucose concentration

The glucose concentration in the unknown sample was determined by:

1. Centrifuge the yeast sample at 10 000rpm for 5 min.
2. Decant the supernatant into a fresh Eppendorf tube and centrifuge at 10 000rpm for 5 min.
3. Dilute in a case where the glucose concentration is not within the linear range (< 1g/l glucose concentration) and record the dilution factor.
4. Repeat steps 3 – 8 in the standard curve determination method replacing the glucose sample with the unknown sample where appropriate.
5. Once the absorbance has been obtained, the glucose concentration of the unknown sample is calculated by using Equation B4.

B.6.2 REPRODUCIBILITY

The coefficient of variance for replicate samples with a glucose concentration of 0.63g/l is 1.7%.

APPENDIX C: STATISTICAL ANALYSIS OF DATA

APPENDIX C1 - EXPERIMENTAL VARIATIONS

Anova: Two-Factor Without Replication for yeast collected on different days

Sampling time (h)	Count	Sum	Average	Variance
0	2	195	97.6	0.045
1	2	194	96.9	0.011
3	2	196	98.0	0.216
4	2	196	98.1	0.007
6	2	195	97.6	0.027
Day 1	5	488	97.6	0.207
Day 2	5	488	97.7	0.307

ANOVA

Source of Variation	SS	df	MS	F	P-value	F_{crit}	Confidence Level (%)
Rows (Sampling time)	1.77	4	0.441	6.08	0.054	6.39	94.6
Columns (Experiments)	0.015	1	0.015	0.207	0.673	7.71	32.7
Error	0.291	4	0.073				
Total	2.07	9					

Anova: Two-Factor Without Replication for Yeast Generations

Scenario 1

Sampling time (h)	Count	Sum	Average	Variance
0	2	190	94.9	1.07
1	2	185	92.5	8.64
4	2	194	97.2	0.108
6	2	191	95.3	1.92
8	2	183	91.6	4.72
Generation 1	5	470	94.0	6.07
Generation 4	5	473	94.6	7.75

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit	Confidence Level (%)
Rows (Sampling times)	39.6	4	9.91	2.53	0.195	6.39	80.5
Columns (Generation)	0.785	1	0.785	0.201	0.677	7.71	32.3
Error	15.7	4	3.92				
Total	56.1	9					

Appendix C2 - REPRODUCIBILITY OF VIABILITY AND PROTEASE DATA

Reproducibility of Viability data

F-Test Two-Sample for Variances

	Sample 1	*Sample 2*
Mean	93.7	93.1
Variance	0.91	1.21
Observations	5	4
df	4	3
F	0.756	
P(F<=f) one-tail	0.384	
F Critical one-tail	0.152	

t-Test: Two-Sample Assuming Unequal Variances

	Sample 1	*Sample 2*
Mean	93.7	93.1
Variance	0.91	1.21
Observations	5	4
Hypothesized Mean Difference	0	
df	6	
t Stat	0.880	
P(T<=t) one-tail	0.206	
t Critical one-tail	1.94	
P(T<=t) two-tail	0.413	
t Critical two-tail	2.45	

Reproducibility of extracellular protease data

F-Test Two-Sample for Variances

	Sample 1	*Sample 2*
Mean	0.034	0.034
Variance	1.58E-06	2.00E-06
Observations	4	4
df	3	3
F	0.792	
P(F<=f) one-tail	0.426	
F Critical one-tail	0.108	

t-Test: Paired Two Sample for Means

	Sample 1	*Sample 2*
Mean	0.034	0.034
Variance	1.58E-06	2.00E-06
Observations	4	4
Pearson Correlation	0.375	
Hypothesized Mean Difference	0	
df	3	
t Stat	0.333	
P(T<=t) one-tail	0.380	
t Critical one-tail	2.35	
P(T<=t) two-tail	0.761	
t Critical two-tail	3.18	

t-Test: Two-Sample Assuming Equal Variances

	Sample 1	*Sample 2*
Mean	0.034	0.034
Variance	1.58E-06	2.00E-06
Observations	4	4
Pooled Variance	1.79E-06	
Hypothesized Mean Difference	0	
df	6	
t Stat	0.264	
P(T<=t) one-tail	0.400	
t Critical one-tail	1.94	
P(T<=t) two-tail	0.801	
t Critical two-tail	2.45	

APPENDIX C3 - REPRODUCIBILITY OF SMALL-SCALE FERMENTATION DATA

Reproducibility of viability data

Anova: Two-Factor Without Replication

Time	*Count*	*Sum*	*Average*	*Variance*
0	4	389	97.4	1.04
2	4	314	78.5	7.24
4	4	324	81.1	36.1
6	4	335	83.7	3.91
8	4	335	83.7	4.01
24	4	347	86.8	10.2
Sample 1	6	496	82.6	76.3
Sample 2	6	520	86.7	40.3
Sample 3	6	519	86.4	27.9
Sample 4	6	510	85.1	53.9

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows (Time)	867	5	173	20.8	2.91E-06	2.901
Columns (Samples)	62.6	3	20.9	2.50	0.0987	3.287
Error	125	15	8.33			
Total	1055	23				

Reproducibility of biomass concentration data

Anova: Two-Factor Without Replication

Time	*Count*	*Sum*	*Average*	*Variance*
0	4	4.58	1.15	0.027
2	4	3.80	0.950	0.014
4	4	4.64	1.16	0.025
6	4	5.49	1.37	0.006
8	4	5.04	1.26	0.011
24	4	6.25	1.56	0.096
Sample 1	6	7.16	1.19	0.074
Sample 2	6	8.03	1.34	0.127
Sample 3	6	7.35	1.23	0.043
Sample 4	6	7.26	1.21	0.026

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows (Time)	0.886	5	0.177	5.74	0.004	2.90
Columns (Samples)	0.076	3	0.025	0.825	0.500	3.29
Error	0.463	15	0.031			
Total	1.426	23				

Reproducibility of cell dry weight data

Anova: Two-Factor Without Replication

Time	*Count*	*Sum*	*Average*	*Variance*
0	4	14.6	3.65	0.158
2	4	15.3	3.82	0.220
4	4	17.8	4.45	0.297
6	4	16.3	4.07	0.051
8	4	15.6	3.91	0.106
24	4	17.1	4.29	0.228
Sample 1	6	23.2	3.87	0.250
Sample 2	6	24.6	4.10	0.157
Sample 3	6	23.1	3.85	0.110
Sample 4	6	25.8	4.30	0.313

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows (Time)	1.78	5	0.356	2.25	0.102	2.90
Columns (Samples)	0.809	3	0.270	1.71	0.208	3.29
Error	2.37	15	0.158			
Total	4.96	23				

Reproducibility of glucose concentration data

Anova: Two-Factor Without Replication

Time	*Count*	*Sum*	*Average*	*Variance*
0	4	49.3	12.3	0.096
2	4	21.5	5.39	0.093
4	4	4.73	1.18	0.001
6	4	3.84	0.959	0.001
8	4	3.04	0.761	0.002
24	4	2.82	0.705	4.98E-05
1	6	21.4	3.57	21.50
2	6	21.9	3.66	23.47
3	6	20.9	3.48	21.09
4	6	21.1	3.51	20.83

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	434	5	86.8	2769	1.11E-21	2.90
Columns	0.107	3	0.036	1.14	0.364	3.29
Error	0.470	15	0.031			
Total	435	23				

Reproduciblity of budding Index data

Anova: Two-Factor Without Replication

Time	*Count*	*Sum*	*Average*	*Variance*
0	4	0.473	0.118	0.000
2	4	2.78	0.695	0.002
4	4	2.36	0.591	0.005
6	4	2.09	0.522	0.001
8	4	1.96	0.491	0.001
24	4	1.32	0.331	0.000
1	6	2.77	0.461	0.042
2	6	2.73	0.455	0.049
3	6	2.70	0.450	0.047
4	6	2.79	0.466	0.036

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.8426	5	0.169	86.2	1.66E-10	2.90
Columns	0.0008	3	0.000	0.141	0.934	3.29
Error	0.0293	15	0.002			
Total	0.8728	23				

Reproducibility of carbon dioxide formation data

Anova: Two-Factor Without Replication

Time	*Count*	*Sum*	*Average*	*Variance*
2	4	6.60	1.65	0.020
4	4	12.0	3.00	0.129
6	4	13.3	3.32	0.180
8	4	14.4	3.60	0.225
24	4	15.9	3.97	0.308
1	5	14.0	2.80	0.455
2	5	17.5	3.51	0.933
3	5	14.2	2.84	0.690
4	5	16.4	3.27	1.29

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	12.6	4	3.16	46.3	3.37E-07	3.26
Columns	1.77	3	0.590	8.65	0.003	3.49
Error	0.818	12	0.068			
Total	15.2	19				

Anova: Two-Factor Without Replication for Untreated yeast samples

Particle size (μm)	*Count*	*Sum*	*Average*	*Variance*
0.0582	4	0.0004	0.0001	0.0000
0.0679	4	0.0012	0.0003	0.0000
0.0791	4	0.0029	0.0007	0.0000
0.0921	4	0.0066	0.0016	0.0000
0.1073	4	0.0153	0.0038	0.0000
0.1250	4	0.0352	0.0088	0.0000
0.1456	4	0.0774	0.0193	0.0000
0.1697	4	0.1594	0.0399	0.0001
0.1977	4	0.3034	0.0759	0.0001
0.2303	4	0.5235	0.1309	0.0001
0.2683	4	0.7858	0.1965	0.0001
0.3125	4	0.9643	0.2411	0.0003
0.3641	4	0.9433	0.2358	0.0005
0.4242	4	0.7886	0.1972	0.0006
0.4941	4	0.6389	0.1597	0.0006
0.5757	4	0.5368	0.1342	0.0007
0.6707	4	0.4977	0.1244	0.0006
0.7813	4	0.5915	0.1479	0.0006
0.9103	4	0.7740	0.1935	0.0005
1.060	4	0.9295	0.2324	0.0004
1.235	4	1.063	0.2658	0.0004
1.439	4	1.143	0.2858	0.0003
1.677	4	1.120	0.2799	0.0003
1.953	4	1.145	0.2863	0.0004
2.276	4	1.516	0.3790	0.0003
2.651	4	2.409	0.6023	0.0001
3.089	4	4.172	1.043	0.0001
3.598	4	6.832	1.708	0.0010
4.192	4	10.83	2.707	0.0044
4.884	4	16.35	4.087	0.0127
5.690	4	23.61	5.902	0.0411
6.628	4	32.06	8.015	0.1171
7.722	4	39.64	9.911	0.2391
8.996	4	43.61	10.90	0.3343
10.48	4	43.94	10.98	0.4784
12.21	4	41.26	10.31	0.0247
14.22	4	34.69	8.672	0.0055
16.57	4	27.71	6.928	0.0466
19.31	4	20.95	5.238	0.1020
22.49	4	14.92	3.729	0.1342
26.20	4	9.949	2.487	0.1282
30.53	4	6.155	1.539	0.0945
35.56	4	3.484	0.8709	0.0544
41.43	4	1.538	0.3845	0.0687
48.27	4	0.3451	0.0863	0.0059
56.23	4	0.0158	0.0039	0.0001
65.51	4	0.0000	0.0000	0.0000
76.32	4	0.0000	0.0000	0.0000
88.91	4	0.0113	0.0028	0.0000
103.6	4	0.0729	0.0182	0.0013
120.7	4	0.1333	0.0333	0.0044
140.6	4	0.1751	0.0438	0.0077
163.8	4	0.1870	0.0467	0.0087
190.8	4	0.1680	0.0420	0.0071
222.3	4	0.1230	0.0307	0.0038
259.0	4	0.0780	0.0195	0.0015
301.7	4	0.0330	0.0083	0.0003
Sample 1	64	100	1.563	9.101
Sample 2	64	100	1.562	9.534
Sample 3	64	100	1.563	8.798
Sample 4	64	100	1.562	10.13

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows (within samples)	2361	63	37.47	1220	2.37E-218	1.382
Columns (between samples)	1.069E-11	3	3.56E-12	1.16E-10	1.000	2.652
Error	5.805	189	0.0307			
Total	2367	255				

Anova: Two-Factor Without Replication for treated yeast samples

Particle size (μm)	*Count*	*Sum*	*Average*	*Variance*
0.0582	2	0.0003	0.0002	0.0000
0.0679	2	0.0011	0.0006	0.0000
0.0791	2	0.0035	0.0017	0.0000
0.0921	2	0.0111	0.0055	0.0000
0.1073	2	0.0351	0.0176	0.0000
0.1250	2	0.1059	0.0529	0.0000
0.1456	2	0.2880	0.1440	0.0001
0.1697	2	0.6928	0.3464	0.0001
0.1977	2	1.472	0.7358	0.0000
0.2303	2	2.778	1.389	0.0003
0.2683	2	4.633	2.317	0.0046
0.3125	2	6.672	3.336	0.0181
0.3641	2	8.262	4.131	0.0372
0.4242	2	9.253	4.626	0.0521
0.4941	2	10.07	5.036	0.0638
0.5757	2	10.66	5.329	0.0690
0.6707	2	10.47	5.237	0.0574
0.7813	2	10.04	5.022	0.0415
0.9103	2	8.838	4.419	0.0222
1.060	2	7.635	3.818	0.0099
1.235	2	6.424	3.212	0.0036
1.439	2	5.270	2.635	0.0011
1.677	2	4.147	2.074	0.0002
1.953	2	3.116	1.558	0.0000
2.276	2	2.310	1.155	0.0001
2.651	2	1.782	0.8909	0.0005
3.089	2	1.205	0.6023	0.0010
3.598	2	1.384	0.6919	0.0030
4.192	2	1.964	0.9820	0.0084
4.884	2	2.993	1.497	0.0204
5.690	2	4.512	2.256	0.0414
6.628	2	6.505	3.252	0.0751
7.722	2	9.026	4.513	0.1477
8.996	2	10.86	5.432	0.1543
10.48	2	11.32	5.660	0.1014
12.21	2	10.31	5.153	0.0352
14.22	2	8.418	4.209	0.0035
16.57	2	6.319	3.159	0.0007
19.31	2	4.406	2.203	0.0038
22.49	2	2.851	1.426	0.0037
26.20	2	1.691	0.8454	0.0021
30.53	2	0.8870	0.4435	0.0011
35.56	2	0.3710	0.1855	0.0005
41.43	2	0	0	0
48.27	2	0	0	0
56.23	2	0	0	0
65.51	2	0	0	0
76.32	2	0	0	0
88.91	2	0	0	0
103.6	2	0	0	0
120.7	2	0	0	0
140.6	2	0	0	0
163.8	2	0	0	0
190.8	2	0	0	0
222.3	2	0	0	0
259.0	2	0	0	0
301.7	2	0	0	0
351.5	2	0	0	0
409.4	2	0	0	0
Sample 1	64	100	1.562	3.673
Sample 2	64	100	1.563	3.697

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows (within samples)	463.3	63	7.354	470.3	2.69E-67	1.518
Columns (between samples)	1.94E-11	1	1.94E-11	1.24E-09	1.000	3.993
Error	0.9852	63	0.0156			
Total	464.3	127				

Anova: Two-Factor Without Replication for yeast concentration of 36.1% wet weight using Rushton turbine at an agitation rate of 800 rpm at 14 °C

Time (s)	*Count*	*Sum*	*Average*	*Variance*
0	3	0.000	0.000	0.00E+00
10	3	0.743	0.248	1.04E-03
20	3	1.02	0.340	2.10E-04
30	3	1.03	0.343	2.77E-04
40	3	1.06	0.354	1.30E-05
50	3	1.06	0.354	5.03E-05
60	3	1.11	0.371	6.63E-05
70	3	1.07	0.357	4.08E-04
80	3	1.06	0.352	8.63E-05
90	3	1.07	0.355	1.75E-04
100	3	1.05	0.350	5.30E-04
110	3	1.04	0.345	3.03E-05
120	3	1.08	0.358	1.08E-04
130	3	1.07	0.357	1.08E-04
140	3	1.12	0.374	2.40E-04
150	3	1.08	0.361	2.00E-04
160	3	1.07	0.356	1.60E-04
170	3	1.09	0.364	6.30E-05
180	3	1.11	0.371	6.03E-05
190	3	1.10	0.366	1.30E-05
200	3	1.08	0.360	1.90E-04
210	3	1.06	0.352	8.33E-06
220	3	1.07	0.358	5.77E-04
230	3	1.05	0.348	2.58E-04
240	3	1.10	0.367	3.67E-04
250	3	1.06	0.354	3.63E-05
260	3	1.05	0.349	8.63E-05
270	3	1.03	0.345	1.70E-04
280	3	1.07	0.358	7.63E-05
290	3	1.05	0.350	4.60E-04
300	3	1.00	0.334	4.20E-04
310	3	1.06	0.354	5.03E-05
320	3	1.00	0.332	2.58E-04
330	3	1.06	0.353	8.63E-05
340	3	1.02	0.340	3.03E-05
350	3	1.03	0.343	8.63E-05
360	3	1.00	0.334	6.20E-04
370	3	1.01	0.335	1.46E-03
380	3	1.05	0.348	5.83E-05
390	3	1.05	0.351	3.50E-04
400	3	1.01	0.337	3.58E-04
410	3	1.01	0.338	1.56E-04
420	3	1.06	0.353	6.33E-06
430	3	1.04	0.347	7.63E-05
440	3	1.02	0.338	8.33E-06
450	3	1.09	0.364	9.30E-05
460	3	1.06	0.352	3.07E-04
470	3	1.04	0.347	2.96E-04
480	3	1.00	0.334	2.83E-04
490	3	1.02	0.341	2.40E-04
500	3	0.98	0.328	3.16E-04
510	3	1.03	0.342	2.58E-04
520	3	0.98	0.327	6.70E-05
530	3	1.02	0.340	8.03E-05
540	3	1.03	0.344	6.30E-05
550	3	1.06	0.353	1.28E-03
560	3	1.05	0.351	6.30E-04
570	3	1.03	0.342	2.77E-04
580	3	1.00	0.333	1.06E-03
590	3	1.04	0.347	2.58E-04
600	3	1.11	0.371	8.63E-05
Sample 1	61	21.0	0.344	2.51E-03
Sample 2	61	20.8	0.341	2.42E-03
Sample 3	61	20.8	0.342	2.41E-03

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows (Time profile)	0.4094	60	0.0068	26.4	2.00E-47	1.43
Columns (between samples)	0.0004	2	0.0002	0.744	0.477	3.07
Error	0.0310	120	0.0003			
Total	0.4408	182				

Anova: Two-Factor With Replication for mixing experiments with and without a nitrogen blanket for yeast concentration of 39.0% wet weight using Rushton turbine at an agitation rate of 400 rpm & 14°C

SUMMARY

	Nitrogen blanket	Open to atmosphere	Total
Sample 1			
Count	61	61	122
Sum	22.6	23.9	46.4
Average	0.3700	0.3911	0.3806
Variance	0.0047	0.0052	0.0050
Sample 2			
Count	61	61	122
Sum	23.9	23.3	47.2
Average	0.3920	0.3823	0.3871
Variance	0.0044	0.0052	0.0048
Sample 3			
Count	61	61	122
Sum	23.3	24.5	47.8
Average	0.3825	0.4016	0.3920
Variance	0.0048	0.0061	0.0055
Total			
Count	183	183	
Sum	69.8	71.7	
Average	0.3815	0.3917	
Variance	0.0047	0.0055	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample (replicates)	0.0081	2	0.0040	0.799	0.4507	3.02
Columns (blanket)	0.0096	1	0.0096	1.89	0.1704	3.87
Interaction (replicates/blanket)	0.0182	2	0.0091	1.79	0.1678	3.02
Within	1.82	360	0.0051			
Total	1.86	365				

APPENDIX D: YEAST HISTORY

Date	Generation No.	Consistency Wet weight (%)	Storage time (h)	pH	Viability @ beginning (%)
17/10/01	4	62.2	1	5.4	96.2
18/10/01	2	62.0	1	5.4	95.7
23/10/01	2	62.0	1	5.4	95.7
24/10/01	2	62.0	24	5.4	96.6
30/10/01	2	62.0	2	5.4	96.6
18/02/02	2	62.0	1	5.4	98.1
20/02/02	2	60.0	2	5.4	97.9
09/05/02	1	60.0	2	5.4	95.0
24/05/02	3	62.0	4	5.4	95.0
24/07/02	1	64.0	1	5.4	95.0
17/09/02	4	59.6	2	5.5	95.0
08/10/02	4	38.0	1	5.3	95.0
17/10/02	2	63.6	1	5.2	95.0
05/12/02	3	63.9	3	5.4	93.8
12/12/02	3	62.6	1	5.4	96.2
22/01/03	1	60.7	1	5.4	94.2
29/01/03	4	41.8	4	5.4	97.2
12/02/03	2	63.1	1	5.4	95.6
19/02/03	2	55.0	4	5.4	96.3
06/03/03	4	46.1	1	5.4	94.4
19/05/03	4	67.0	2	5.4	97.7
22/05/03	7	58.4	3	5.2	97.6
26/05/03	4	51.8	2	5.4	97.0
29/05/03	4	53.1	2	5.4	97.2
09/06/03	2	57.0	1	5.4	96.5
13/06/03	5	54.0	2	5.5	97.4
17/06/03	2	60.3	1	5.4	95.6
19/06/03	5	60.0	4	5.4	95.0
03/07/03	2	78.4	1	5.4	95.6
31/07/03	4	61.2	2	5.4	92.3
01/08/03	4	60.9	2	5.4	96.9
05/08/03	4	54.4	1	5.4	96.2
06/08/03	0	65.1	3	5.4	97.6
07/08/03	4	59.1	1	5.4	98.1

APPENDIX E: CALIBRATION OF INSTRUMENTS

E.1 DENSITOMETER

1. Set "Display Select" to T
2. Set "Sampling Rate" to 2
3. Measure T H_2O after 15 minutes
4. Rinse U-tube with alcohol
5. Dry with pump
6. Switch off pump
7. Read T air at 20°C
8. Calculate constants A and B using Equations E1 and E2
9. Programme Densitometer with constants A and B
10. Check constants A and B using "Display Select" in Q

E.2 CONDUCTIVITY METER

The conductivity of samples of 0.001, 0.01, 0.1 and 1M KCl at 0, 18 and 25°C were obtained from literature (Lide and Frederikse, Shugar and Dean, 1990) and this was compared with those obtained from literature. These measurements were done in triplicates and the average, standard deviation and coefficient of variance were calculated. The coefficients of variance at the lower concentrations (0.001 and 0.01M KCl) were about a 10^{th} of those obtained at the higher concentration range (Table E1).

Table E1: Calibration data for conductivity meter

0.001M KCl							
Temperature	Conductivity (μS/cm)						Coefficient of variance
(°C)	Theory	Sample 1	Sample 2	Sample 3	Average	Stdev	(%)
0	75.0	82.0	84.0	83.8	83.3	1.1	1.3
18	127.0	156.1	128.6	131.1	138.6	15.2	11.0
25	147.0	180.3	149.3	154.9	161.5	16.5	10.2
0.01M KCl							
Temperature	Conductivity (μS/cm)						Coefficient of variance
(°C)	Theory	Sample 1	Sample 2	Sample 3	Average	Stdev	(%)
0	773	760	730	770	753	21	2.8
18	1220	1349	1194	1209	1251	85	6.8
25	1408	1558	1375	1390	1441	102	7.1
0.1M KCl							
Temperature	Conductivity (mS/cm)						Coefficient of variance
(°C)	Theory	Sample 1	Sample 2	Sample 3	Average	Stdev	(%)
0	7.13	6.99	7.04	6.93	6.99	0.06	0.8
18	11.16	10.86	10.96	10.81	10.88	0.08	0.7
25	12.85	12.52	12.64	12.40	12.52	0.12	1.0
1M							
Temperature	Conductivity (mS/cm)						Coefficient of variance
(°C)	Theory	Sample 1	Sample 2	Sample 3	Average	Stdev	(%)
0	65.1	63.0	62.9	63.1	63.0	0.1	0.2
18	97.8	96.5	95.2	94.7	95.5	0.9	1.0
25	111.3	111.3	107.0	107.5	108.6	2.4	2.2